SPECIFICATION

HYPERTHERMOSTABLE PROTEASE GENE

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TECHNICAL FIELD

The present invention relates to a hyperthermostable protease useful as an industrial enzyme, a gene encoding the same and a method for preparation of the enzyme by the genetic engineering.

BACKGROUND ART

The proteases are the enzymes which cleave peptide bonds in the proteins, and a number of the proteases have been found in animals, plants and microorganisms. They are used not only as reagents for research works and medical supplies, but also in industrial fields such as additives for detergents, food processing and chemical synthesis utilizing the reverse reactions, and it can be said that they are very important enzymes from an industrial viewpoint. For proteases to be used in industrial fields, since very high physical and chemical stabilities are required, in particular, enzymes having high thermostabilities are preferred to use. At present, proteases predominantly used in industrial fields are those produced by bacteria of the genus <u>Bacillus</u> because they have relatively high thermostability.

However, enzymes having further superior properties are desired and activities have been attempted to obtain enzymes from microorganisms which grow at high temperature, for example, thermophiles of the genus Bacillus.

On the other hand, a group of microorganisms, named as hyperthermophiles, are well adapted themselves to high temperature environments and therefor they are expected to be a source supplying various thermostable enzymes. It has been known that one of these hyperthermophiles, Pyrococcus furiosus, produces proteases [Appl. Environ. Microbiol., volume 56, page 1992-1998 (1990), FEMS Microbiol. Letters, volume 71, page 17-20 (1990), J. Gen. Microbiol., volume 137, page 1193-1199 (1991)].

A hyperthermophile belonging to the genus Pyrococcus, Pyrococcus sp. Strain KOD1 is reported to produce a thiol protease (cysteine protease) [Appl. Environ. Microbiol., volume 60, page 4559-4566 (1994)]. Bacteria belonging to the genus Thermococcus, Staphylothermus and Thermobacteroides, which are also hyperthermophiles, are known to produce a protease [Appl. Microbiol. Biotechnol., volume 34, page 715-719 (1991)].

OBJECTS OF THE INVENTION

As the proteases produced by these hyperthermophiles have high thermostabilities, they are expected to be applicable to new applications to which any known enzymes has not been utilized. However, the above publication merely teach that thermostable protease activities present in cell-free extract or crude enzyme solution obtained from culture supernatant, and there is no disclosure about properties of isolated and purified enzymes and the like. Only a protease produced by strain KOD1 is obtained as the purified form. However, since a cysteine protease has the defect that it easily loses the activity by oxidation, it is disadvantageous in the industrial use. In addition, since a cultivation of microorganisms at high temperature is required to obtain enzymes from these hyperthermophiles, there is a problem in industrial production of the enzymes.

In order to solve the above problems, an object of the present invention is to provide a protease of the hyperthermophiles which is advantageous in the industrial use, to isolate a gene encoding a protease of the hyperthermophiles, and to provide a method for preparation of a hyperthermostable protease using the gene by the genetic engineering.

DISCLOSURE OF THE INVENTION

In order to obtain a hyperthermostable protease gene, the present inventors originally tried to purify a protease from microbial cells and a culture supernatant of Pyrococcus furiosus DSM3638 so as to determine a partial amino acid sequence of the enzyme. However, purification of the protease was very difficult in either cases of using the microbial cells or the culture supernatant, and the present inventors failed to obtain such an enzyme sample having sufficient purity for determination of its partial amino acid sequence.

As a method for cloning a gene for an objective enzyme without any information about a primary structure of the enzyme protein, there is an expression cloning method. For example, a pullulanase gene originating in Pyrococcus woesei (WO92/02614) has been obtained according to this method. However, in an expression cloning method, a plasmid vector is generally used and, in such case, it is necessary to use restriction enzymes which can cleave an objective gene into relatively small DNA fragments so that the fragments can be inserted into the plasmid vector without cleavage of any internal portion of the objective gene. Therefore, the expression cloning method is not always applicable to cloning of all kind of enzyme genes. Furthermore, it is necessary to test for an enzyme activity of a large number of clones and this operation is

complicated.

The present inventors have attempted to isolate a protease gene by using a cosmid vector which can maintain a larger DNA fragment (30-50kb) instead of a plasmid vector to prepare a cosmid library of Pyrococcus furiosus genome and investigating cosmid clone in the library to find out a clone expressing a protease activity. By using the cosmid vector, the number of transformants to be screened can be reduced in addition to lowering of possibilities of cleavage of a internal portion of the enzyme gene. On the other hand, since the copy number of a cosmid vector in a host cell is not higher than that of a plasmid vector, it may be that an amount of the enzyme expressed is too small to detect it.

In view of high thermostability of the objective enzyme, firstly, the present inventors have cultured respective transformants in a cosmid library, separately, and have combined this step with a step for preparing lysates containing only thermostable proteins from the microbial cells thus obtained, and used these lysates for detecting the enzyme activity. Further, the use of the gelatin-containing SDS-polyacrylamide gel electrophoresis for detecting the protease activity allowed the detection of a trace amount of the enzyme activity.

Thus, the present inventors obtained several

cosmid clones expressing the protease activity from the cosmid library of Pyrococcus furiosus and successfully isolated a gene encoding a protease from the inserted DNA fragment contained in the clones. In addition, the present inventors confirmed that a protease encoded by the gene has the extremely high thermostability.

By comparing an amino acid sequence of the hyperthermostable protease deduced from the nucleotide sequence of the gene with amino acid sequences of known proteases originating in microorganisms, homology of the amino acid sequence of the front half portion of the hyperthermostable protease with those of a group of alkaline serine proteases, a representative of which is subtilisin, has been shown. In particular, the extremely high homology has been found at each region around the four amino acid residues which are known to be important for the catalytic activity of the enzyme. Thus, since the protease produced by Pyrococcus furiosus, which is active at such a high temperature that proteases originating in mesophiles are readily inactivated, has been shown to retain a structure similar to those of enzymes from mesophiles, it has been suggested that similar proteases would also be produced by hyperthermophiles other than Pyrococcus furiosus.

Then, the present inventors have noted

possibilities that, in the nucleotide sequence of the hyperthermostable protease gene obtained, the nucleotide sequence encoding regions showing high homology with subtilisin and the like can be used as a probe for detecting hyperthermostable protease gene, and have attempted to detect protease genes originating in hyperthermophiles by PCR using synthetic DNAs designed based on the nucleotide sequences as primers so as to clone the gene. As a result, it was found that a fragment of gene having the homology with the above gene existed in a hyperthermophile, <u>Thermococcus</u> <u>celer</u> DSM2476. The cloning of the full length of the gene was difficult and this was thought to be due to that the product derived from the gene was harmful to the host.

The present inventors used <u>Bacillus</u> <u>subtilis</u> as a host for cloning and found that harbouring of the full length gene was possible and the expressed protease was extracellularly secreted, further revealed that the expressed protease showed the protease activity at 95 °C and had the high thermostability. Upon this, the molecular weight of a protease encoded by the gene was found to be less than half of that of the high-molecular protease derived from the <u>Pyrococcus</u> <u>furiosus</u> described above.

In addition, by hybridization using a fragment of the gene as a probe, we found that the second protease gene

different from that of the high-molecular protease was present in Pyrococcus furiosus. The gene encodes a protease having a similar molecular weight to that of the hyperthermostable protease derived from Thermococcus celer, and the gene was isolated and introduced into Bacillus subtilis and, thereby, a product expressed from the gene was extracellularly secreted. The expressed protease showed the enzyme activity at 95 °C and had the high thermostability. In addition, the amino acid sequence of a mature protease produced by processing of the protease was revealed.

As these two kinds of proteases are extracellularly secreted without any special procedures, it is thought that a signal peptide encoded by the gene itself functions in Bacillus subtilis. The amount of expressed both proteases per culture is remarkably higher as compared with the high-molecular protease derived from Pyrococcus furiosus which is expressed in Escherichia coli or Bacillus subtilis. In addition, when the gene is expressed by utilizing a promoter of the subtilisin gene and a signal sequence, the amount of the expressed protease was further increased.

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~~Furthermore, the present inventors made a gene encoding a hybrid protease which was a chimera protein from both proteases, and confirmed that an enzyme expressed by~~

~~the gene showed the protease activity under high temperature conditions as the above hyperthermostable protease which resulted in the completion of the present inventior.~~

SUMMARY OF THE INVENTION

The first aspect of the present invention provides a hyperthermostable protease having the amino acid sequence described in SEQ ID No. 1 of the Sequence Listing or functional equivalents thereof as well as a hyperthermostable protease gene encoding the hyperthermostable proteases, inter alia, a hyperthermostable protease gene having the nucleotide sequence described in SEQ ID No. 2 of the Sequence Listing. Further, a gene which hybridizes with this hyperthermostable protease gene and encodes a hyperthermostable protease is also provided.

In addition, the second aspect of the present invention provides a hyperthermostable protease having the amino acid sequence described in SEQ ID No. 3 of the Sequence Listing or functional equivalents thereof as well as a hyperthermostable protease gene encoding the hyperthermostable proteases, inter alia, a hyperthermostable protease gene having the nucleotide sequence described in SEQ ID No. 4 of the Sequence Listing.

Further, a gene which hybridizes with this hyperthermostable protease gene and encodes a hyperthermostable protease is also provided.

In addition, the third aspect of the present invention provides a hyperthermostable protease having the amino acid sequence described in SEQ ID No. 5 of the Sequence Listing or functional equivalents thereof as well as a hyperthermostable protease gene encoding the hyperthermostable proteases, inter alia, a hyperthermostable protease gene having the nucleotide sequence described in SEQ ID No. 6 of the Sequence Listing. Further, a gene which hybridizes with this hyperthermostable protease gene and encodes a hyperthermostable protease is also provided.

Further, the present invention provides a method for preparation of the hyperthermostable protease which comprises cultivating a transformant containing the hyperthermostable protease gene of the present invention, and collecting the hyperthermostable protease from the culture.

As used herein, the term "functional equivalents" means as follows:

It is known that although, among naturally-occurring proteins, a mutation such as deletion, addition, substitution and the like of one or a few (for

example, up to 5% of the whole amino acids) amino acid(s) can occur in the amino acid sequence thereof due to the modification reaction and the like of the produced proteins in the living body or during purification besides the polymorphism or mutation of the genes encoding them, there are proteins, in spite of the mutation described above, showing the substantially equivalent physiological or biological activity to that of the proteins having no mutation. When the proteins have the slight difference in the structures and, nevertheless, the great difference in the functions thereof is not recognized, they are called functional equivalents. This is true when the above mutations are artificially introduced into the amino acid sequence of the proteins and, in this case, further a more variety of mutants can be made. For example, a polypeptide where a certain cysteine residue is replaced with serine residue in the amino acid sequence of human interleukin-2 (IL-2) shows the interleukin-2 activity [Science, volume 224, page 1431 (1984)].

A product of the gene which is transcribed and translated from the hyperthermostable protease gene of the present invention is an enzyme precursor (preproenzyme) containing two regions, one of them is a signal peptide necessary for extracellular secretion and the other is a propeptide which is removed upon expression of the

activity. When a transformant to which the above gene has been transferred can cleave this signal peptide, an enzyme precursor (proenzyme) from which the signal peptide has been removed is extracellularly secreted. Further, an active form enzyme from which the propeptide has been removed is produced by the self-digestion reaction between proenzymes. All of the preproenzyme, proenzyme and active form enzyme thus obtained from the gene of the present invention are proteins which finally have the equivalent function and fall within the scope of "functional equivalents".

As apparent to those skilled in the art, an appropriate signal peptide may be selected depending upon a host used for the expression of a gene of interest. The signal peptide may be removed when the extracellular secretion is not desired. Therefore, among hyperthermostable protease genes disclosed herein, the genes from which a portion encoding a signal peptide has been removed, and the genes where the portion is replaced with other nucleotide sequence are also within the scope of the present invention in the context that they encode the proteases showing the essentially equivalent activity.

As used herein, a gene which "hybridizes to a hyperthermostable protease gene" refers to a gene which hybridizes with the hyperthermostable protease gene under

the stringent conditions, that is, those where incubation is carried out at 50 °C for 12 to 20 hours in 6 × SSC (1 × SSC represents 0.15M NaCl, 0.015M sodium citrate, pH7.0) containing 0.5% SDS, 0.1% bovine serum albumin (BSA), 0.1% polyvinylpyrrolidone, 0.1% Ficoll 400 and 0.01% denatured salmon sperm DNA.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 is a figure showing a restriction map of a DNA fragment derived from Pyrococcus furiosus contained in the plasmid pTPR12 and the plasmid pUBP13.

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Fig. 2 is a figure showing a design of the oligonucleotide PRO-1F.

Fig. 3 is a figure showing a design of the oligonucleotide PRO-2F and PRO-2R.

Fig. 4 is s figure showing a design of the oligonucleotide PRO-4R.

Fig. 5 is a restriction map of the plasmid p2F-4R.

Fig. 6 is a restriction map of the plasmid pTC3.

Fig. 7 is a restriction map of the plasmid pTCS6.

Fig. 8 is a restriction map of the plasmid pTC4.

Fig. 9 is a figure showing the procedures for constructing the plasmid pSTC3.

Fig. 10 is a restriction map of the plasmid pSTC3.

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Fig. 11 is s figure comparing the amino acid

sequences of the various proteases.

Fig. 12 is a continuation of Fig. 11.

Fig. 13 is a figure showing a restriction map around the protease PFUS gene on the _Pyrococcus furiosus_ chromosomal DNA.

Fig. 14 is a restriction map of the plasmid pSPT1.

Fig. 15 is a restriction map of the plasmid pSNP1.

Fig. 16 is a restriction map of the plasmid pPS1.

Fig. 17 is a restriction map of the plasmid pNAPS1.

Fig. 18 is a figure showing the optimum temperature for the enzyme preparation TC-3.

Fig. 19 is a figure showing the optimum temperature for the enzyme preparation NAPS-1.

Fig. 20 is a figure showing the optimum pH for the enzyme preparation TC-3.

Fig. 21 is a figure showing the optimum pH for the enzyme preparation NP-1.

Fig. 22 is a figure showing the optimum pH for the enzyme preparation NAPS-1.

Fig. 23 is a figure showing the thermostability of the enzyme preparation TC-3.

Fig. 24 is a figure showing the thermostability of the enzyme preparation NP-1.

Fig. 25 is a figure showing the thermostability of

the activated enzyme preparation NP-1.

Fig. 26 is a figure showing the thermostability of the enzyme preparation NAPS-1.

Fig. 27 is a figure showing the pH-stability of the enzyme preparation NP-1.

Fig. 28 is a figure showing the stability of the enzyme preparation NP-1 in the presence of SDS.

Fig. 29 is a figure showing the stability of the enzyme preparation NAPS-1 in the presence of SDS.

Fig. 30 is a figure showing the stability of the enzyme preparation NAPS-1 in the presence of acetonitrile.

Fig. 31 is a figure showing the stability of the enzyme preparation NAPS-1 in the presence of urea.

Fig. 32 is a figure showing the stability of the enzyme preparation NAPS-1 in the presence of guanidine hydrochloride.

PREFERRED EMBODIMENTS OF THE INVENTION

The hyperthermostable protease gene of the present invention can be obtained by screening the gene library of hyperthermophiles. As the hyperthermophile, bacteria belonging to the genus Pyrococcus can be used and the gene of interest can be obtained by screening a cosmid library of Pyrococcus furiosus genome.

For example, Pyrococcus furiosus DSM3638 can be

used as Pyrococcus furiosus, and the strain is available from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

One example of the cosmid libraries of Pyrococcus furiosus genome can be obtained by ligating DNA fragments which are obtained by partial digestion of the genomic DNA of Pyrococcus furiosus DSM3638 with a restriction enzyme Sau3A1 (manufactured by Takara Shuzo Co., Ltd.), with the triple helix cosmid vector (manufactured by Stratagene), and packaging the ligated product into a lambda phage particle according to the in vitro packaging method. Then, the library is transduced into the suitable Escherichia coli, for example, Escherichia coli DH5αMCR (manufactured by BRL) to obtain the transformants, followed by cultivation them, collecting the microbial cells, subjecting them to heat treatment (for example, 100 °C for 10 minutes), sonicating and subjecting them to heat treatment (for example, 100 °C for 10 minutes) again. The presence or absence of the protease activity in the resulting lysate can be screened by utilizing the gelatin--containing SDS-polyacrylamide gel electrophoresis.

In this manner, a cosmid clone containing a hyperthermostable protease gene expressing a protease which is resistant to the above heat treatment can be obtained.

Further, the cosmid DNA prepared from the cosmid clone thus obtained can be digested into fragments with a suitable restriction enzyme to obtain a recombinant plasmid with each fragment incorporated. Then, a suitable microorganism is transformed with the plasmid, and the protease activity expressed by the resulting transformant can be examined to obtain a recombinant plasmid containing a hyperthermostable protease gene of interest.

That is, the cosmid prepared from one of the above cosmid clones is digested with NotI and PvuII (both manufactured by Takara Shuzo Co., Ltd.) to give an about 7.5kb DNA fragment which can be isolated and inserted between the NotI site and the SmaI site of the plasmid vector pUC19 (manufactured by Takara Shuzo Co., Ltd.) into which the NotI linker (manufactured by Takara Shuzo Co., Ltd.) has been introduced. The plasmid was designated the plasmid pTPR12 and Escherichia coli JM109 transformed with the plasmid was designated Escherichia coli JM109/pTPR12 and has been deposited at National Institute of Bioscience and Human-Technology at 1-1-3. Higashi, Tsukuba-shi, Ibaraki-ken, Japan since May 24, 1994 (original deposit date) as the accession number FERM BP-5103 under Budapest Treaty.

The lysate of the Escherichia coli JM109/pTPR12 shows the similar protease activity to that of the above

cosmid clone on the gelatin-containing SDS-polyacrylamide gel.

The nucleotide sequence of the DNA fragment, derived from Pyrococcus furiosus, which was inserted into the plasmid pTPR12 can be determined by a conventional method, for example, the dideoxy method. The nucleotide sequence of the 4.8kb portion flanked by two DraI sites within the DNA fragment inserted into the plasmid pTPR12 is shown in SEQ ID No. 7 of the Sequence Listing. The amino acid sequence of a gene product deduced from the nucleotide sequence is shown in SEQ ID No. 8 of the Sequence Listing. Thus, a hyperthermostable protease, the nucleotide sequence and the amino acid sequence of which were revealed, derived from Pyrococcus furiosus was designated the protease PFUL. As shown in SEQ ID No. 8 of the Sequence Listing, the protease PFUL is a protease consisting of 1398 residues and having a high-molecular weight of more than 150 thousands.

The protease PFUL gene can be expressed using Bacillus subtilis as a host. As Bacillus subtilis, Bacillus subtilis DB104 can be used and the strain is the known one described in Gene, volume 83, page 215-233 (1989). As a cloning vector, the plasmid pUB18-P43 can be used and the plasmid was gifted from Dr. Sui-Lam Wong at Calgary University. The plasmid contains the kanamycin resistant gene as a selectable marker.

There is the plasmid pUBP13 where an about 4.8kb DNA fragment obtained by digestion of the plasmid pTPR13 with DraI has been inserted into the SmaI site of the plasmid vector pUB18-P43. In the plasmid, the protease PFUL gene is located downstream of the P43 promoter [J. Biol. Chem., volume 259, page 8619-8625 (1984)] which functions in Bacillus subtilis. Bacillus subtilis DB104 transformed with the plasmid was designated Bacillus subtilis DB104/pUBP13. The lysate of the transformant shows the similar protease activity to that of the Escherichia coli JM109/pTPR12.

However, only a trace amount of the protease activity is detected in a culture supernatant of the transformant. This is thought to be due to that a molecular weight of the protease PFUL is extremely high and it is not translated effectively in Bacillus subtilis, and that a signal sequence encoded by the protease PFUL gene dose not function well in Bacillus subtilis. There is a possibility that the protease PFUL is a membrane-bound type protease, and the peptide chain on the C-terminal side of the protease PFUL may be a region for binding to the cell membrane.

Fig. 1 shows a restriction map around the protease PFUL gene on the Pyrococcus furiosus chromosome, as well as a DNA fragment inserted into the plasmid pTPR12 and that

inserted into the plasmid pUBP13. In addition, an arrow in Fig. 1 shows the open reading frame encoding the protease PFUL.

By comparing the amino acid sequence of the protease PFUL represented by SEQ ID. No. 8 of the Sequence Listing with that of a protease derived from the known microorganism, it is seen that there is the homology between the amino acid sequence of the front half portion of the protease PFUL and that of a group of alkaline serine proteases, a representative of which is subtilisin [Protein Engineering, volume 4, page 719-737 (1991)], and that there is the extremely high homology around four amino acid residues which are considered to be important for catalytic activity of the proteases.

As it was revealed that regions commonly present in the proteases derived from a mesophile are conserved in the amino acid sequence of the protease PFUL produced by the hyperthermophile Pyrococcus furiosus, it is expected that these regions are present in the same kind of proteases produced by the hyperthermophiles other than Pyrococcus furiosus.

That is, a DNA having the suitable length can be prepared based on the sequence of a portion encoding the amino acid sequence of a region having the high homology with that of subtilisin and the like, and the DNA can be

used as a probe for hybridization or as a primer for gene amplification such as PCR and the like to screen a hyperthermostable protease gene similar to the present enzyme present in various hyperthermophiles.

In the above method, a DNA fragment containing only a portion of the gene of interest is obtained in some cases. Upon this, the nucleotide sequence of the resulting DNA fragment is investigated and confirmed that it is a portion of the gene of interest and, thereafter, hybridization can be carried out using the DNA fragment or a part thereof as a probe or PCR can be carried out using a primer synthesized based on the nucleotide sequence of the DNA fragment to obtain the whole gene of interest.

The above hybridization can be carried out under the following conditions. That is, a membrane to which a DNA is fixed is incubated with a probe suitably labeled at 50 °C for 12 to 20 hours in 6 × SSC (1 × SSC represents 0.15M NaCl, 0.015M sodium citrate, pH 7.0) containing 0.5% SDS, 0.1% bovine serum albumin (BSA), 0.1% polyvinylpyrrolidone, 0.1% Ficoll 400 and 0.01% denatured salmon sperm DNA. After the completion of incubation, the membrane is washed, beginning with washing at 37 °C in 2 × SSC containing 0.5% SDS, varying the SSC concentration in a range of to 0.1 × and a temperature in a range of to 50 °C, until a signal from a probe hybridized to the fixed DNA

can be discriminated from the background.

In addition, it is apparent to those skilled in the art that a probe and a primer can be made based on the thus obtained new hyperthermostable gene to obtain another hyperthermostable protease gene according to the similar method.

Figs. 2, 3 and 4 show the relationship among the amino acid sequences of regions in the amino acid sequence of the protease PFUL which have high homology with those of subtilisin and the like, the nucleotide sequence of the protease PFUL gene encoding the region, and the nucleotide sequences of the oligonucleotides PRO-1F, PRO-2F, PRO-2R and PRO-4R which were synthesized based thereon. Further, SEQ ID Nos. 9, 10, 11 and 12 of the Sequence Listing show the nucleotide sequences of the oligonucleotides PRO-1F, PRO-2F, PRO-2F and PRO-4R. That is, SEQ ID Nos. 9-12 are the nucleotide sequences of one example of the oligonucleotides used for screening the hyperthermostable protease gene of the present invention.

By using a combination of the oligonucleotides as primer, a protease gene can be detected by PCR using a chromosomal DNA of the various hyperthermophiles as a template.

As the hyperthermophiles, the bacteria belonging to the genus <u>Pyrococcus</u>, genus <u>Thermococcus</u>, genus

Staphylothermus, genus *Thermobacteroides* and the like can be used. As the bacteria belonging to genus *Thermococcus*, for example, *Thermococcus celer* DSM2476 can be used and the strain can be obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. When PCR is carried out using a chromosomal DNA of *Thermococcus celer* DSM2476 as a template and using a combination of the oligonucleotides PRO-1F and PRO-2R or a combination of the oligonucleotides PRO-2F and PRO-4R as a primer, the specific amplification of a DNA fragment is observed and the presence of a protease gene can be identified. In addition, the amino acid sequence encoded by the DNA fragment can be estimated by inserting the DNA fragments into a suitable plasmid vector to make a recombinant plasmid and, thereafter, determining the nucleotide sequence of the inserted DNA fragment by the dideoxy method.

A DNA fragments of about 150 bp amplified using the oligonucleotides PRO-1F and PRO-2R and DNA fragment of about 550bp DNA amplified using the oligonucleotides PRO-2F and PRO-4R are inserted into the HincII site of the plasmid vector pUC18 (manufactured by Takara Shuzo Co., Ltd.). The recombinant plasmids are designated the plasmid p1F-2R(2) and the plasmid p2F-4R, respectively. SEQ ID No. 13 of the Sequence Listing shows the nucleotide sequence of the

inserted DNA fragment in the plasmid p1F-2R(2) and the amino acid sequence deduced therefrom and SEQ ID No. 14 of the Sequence Listing shows the nucleotide sequence of the inserted DNA fragment in the plasmid p2F-4R and the amino acid sequence deduced therefrom. In the SEQ ID No. 13 of the Sequence Listing, the nucleotide sequence from the 1st to the 21st nucleotides and that from the 113rd to he 145th nucleotides and, In the SEQ ID No. 14 of the Sequence Listing, the nucleotide sequence from the 1st to the 32nd nucleotides and that from the 532nd to the 564th nucleoti des are the nucleotide sequence derived from the oligonucleotides used in PCR as primers (each corresponding to the oligonucleotides PRO-1F, PRO-2R, PRO-2F and PRO-4R, respectively). The amino acid sequences having the homology with that of the protease PFUL and the alkaline serine proteases derived from the various microorganisms are present in the amino acid sequences represented by SEQ ID Nos. 13 and 14 of the Sequence Listing, indicating that the above PCR-amplified DNA fragments were amplified with the protease gene as a template.

A restriction map of the plasmid p2F-4R is shown in Fig. 5. In Fig. 5, a thick solid line indicates the DNA fragment inserted into the plasmid vector pUC18.

Then, a hyperthermostable protease gene, for example, a gene of the hyperthermostable protease produced

by Thermococcus celer can be obtained by screening the gene library of hyperthermostable bacteria using above oligonucleotides or the amplified DNA fragments obtained by the above PCR as a probe.

One example of the gene libraries of Thermococcus celer, there is a library prepared by partially digesting a chromosomal DNA of Thermococcus celer DSM2476 with the restriction enzyme Sau3AI to obtain a DNA fragment, ligating the fragment with lambda GEM-11 vector (manufactured by Promega) and packaged it into the lambda phage particle using the in vitro packaging method. Then, the library can be transduced into suitable Escherichia coli, for example, Escherichia coli LE392 (manufactured by Promega) to allow to form the plaques on a plate, and plaque hybridization can be carried out using an amplified DNA fragment obtained by the above PCR as a probe to obtain phage clones containing a gene of interest.

Further, a phage DNA prepared from the phage clones thus obtained can be digested with a suitable restriction enzyme, and southern hybridization can be carried out using the above probe to detect a DNA fragment containing a protease gene.

When the phage DNA prepared from the phage clones obtained by the plaque hybridization is digested with KpnI and BamHI (both manufactured by Takara Shuzo Co., Ltd.), an

about 5kb DNA fragment is hybridized to the probe, and the about 5kb DNA fragment can be isolated and inserted between the KpnI site and the BamHI site of the plasmid vector pUC119 (manufactured by Takara Shuzo Co., Ltd.) to obtain a recombinant plasmid. The plasmid was designated the plasmid pTC3 and <u>Escherichia coli</u> JM109 transformed with the plasmid was designated <u>Escherichia coli</u> JM109/pTC3. A restriction map of the plasmid pTC3 is shown in Fig. 6. In Fig. 6, a thick solid line designates the DNA fragment inserted into the plasmid vector pUC119.

A DNA fragment which does not contain the protease gene within the DNA fragment inserted into the plasmid pTC3 can be removed according to the following procedures. That is, after the plasmid pTC3 is digested with SacI (manufactured by Takara Shuzo Co., Ltd.), southern hybridization is carried out according to the similar procedures described above and it is found that an about 1.9 kb DNA fragment hybridizes to the probe. Then, the about 1.9 kb DNA fragment can be isolated and inserted into the SacI site of the plasmid vector pUC118 (manufactured by Takara Shuzo Co., Ltd.) to make a recombinant vector. The plasmid was designated the plasmid pTCS6 and <u>Escherichia coli</u> JM109 transformed with the plasmid was designated <u>Escherichia coli</u> JM109/pTCS6. A restriction map of the plasmid pTCS6 is shown in Fig. 7. In Fig. 7, a thick solid

line designates the DNA fragment inserted into the plasmid vector pUC118. By determining the nucleotide sequence of the DNA fragment inserted into the plasmid pTCS6 by the dideoxy method, it can be confirmed that a protease gene is present in the DNA fragment. SEQ ID No. 15 of the Sequence Listing shows the nucleotide sequence of the fragment. By comparing the nucleotide sequence with that of the DNA fragment inserted into the plasmid p1F-2R (2) or that of the plasmid p2F-4R represented by SEQ ID No. 13 or 14 of the Sequence Listing, it is seen that the DNA fragment inserted into the plasmid pTCS6 contains the DNA fragment which is also shared by the plasmid p2F-4R but lacks a 5′ region of the protease gene.

Like this, the hyperthermostable protease gene, derived from <u>Thermococcus</u> <u>celer</u>, contained in the plasmid pTCS6 lacks a portion thereof. However, as apparent to those skilled in the art, a DNA fragment covering the full length hyperthermostable protease gene can be obtained by (1) screening the gene library once more, (2) conducting southern hybridization using a chromosomal DNA, or (3) obtaining a DNA fragment of a 5′ upstream region by PCR using a cassette and a cassette primer (Takara Shuzo Co., Ltd., Genetic Engineering Products Guidance, 1994-1995 edition, page 250-251).

The present inventors selected the method (3).

That is, a chromosomal DNA of the Thermococcus celer is completely digested with a few restriction enzymes, followed by ligation with a cassette (manufactured by Takara Shuzo Co., Ltd.) which corresponds to the restriction enzyme used. PCR is carried out using this ligation product as a template and the primer TCE6R (SEQ ID No. 16 of the Sequence Listing shows the nucleotide sequence of the primer TCE6R) and the cassette primer C1 (manufactured by Takara Shuzo Co., Ltd.) as primers. When the above procedures are carried out using the restriction enzyme HindIII (manufactured by Takara Shuzo Co., Ltd.), an about 1.8 kb DNA fragment is amplified, and a DNA fragment of about 1.5 kb which is obtained by digesting above amplified fragment with HindIII and SacI can be inserted into between the HindIII site and the SacI site of the plasmid vector pUC119 to obtain a recombinant plasmid. The plasmid was designated the plasmid pTC4 and Escherichia coli JM109 transformed with the plasmid was designated Escherichia coli JM109/pTC4. A restriction map of the plasmid pTC4 is shown in Fig. 8. In Fig. 8, a thick solid line designates the DNA fragment inserted into the plasmid vector pUC119.

By determining the nucleotide sequence of the DNA fragment inserted into the plasmid pTC4 by the dideoxy method, it can be confirmed that a protease gene is present

in the DNA fragment. SEQ ID No. 17 of the Sequence Listing shows the nucleotide sequence of the fragment. By comparing the amino acid sequence deduced from the nucleotide sequence with those of the various proteases, it is found that the DNA fragment inserted into the plasmid pTC4 covers the 5′ region of the hyperthermostable protease gene which the plasmid pTCS6 lacks. By combining the nucleotide sequence with that of the DNA fragment inserted into the plasmid pTCS6 represented by SEQ ID No. 15 of the Sequence Listing, the nucleotide sequence of the full length hyperthermostable gene derived from <u>Thermococcus celer</u> can be identified. The nucleotide sequence of the open reading frame present in the obtained nucleotide sequence is shown in SEQ ID No. 2 of the Sequence Listing and the amino acid sequence deduced from the nucleotide sequence is shown in SEQ ID No. 1, respectively. Thus, the hyperthermostable protease derived from <u>Thermococcus celer</u>, with the nucleotide sequence encoding it and the amino acid sequence thereof revealed was designated the protease TCES. The full length of the protease TCES gene can be reconstituted by combining the inserted DNA fragment of the plasmid pTC4 and that of the plasmid pTCS6.

It is contemplated that the protease activity expressed by the gene can be confirmed by reconstituting the full length protease TCES gene from two DNA fragments

contained in pTC4 and pTCS6, and inserting this downstream of the lac promoter of a plasmid to give an expression plasmid which is introduced into Escherichia coli. However, this method affords no transformants into which the expression vector of interest has been introduced, and it is predicted that the production of a product expressed from the gene is harmful or lethal to Escherichia coli. It is contemplated that, in such as case, for example, a protease is extracellularly secreted using Bacillus subtilis as a host to confirm the activity.

As a host for expressing the protease TCES gene in Bacillus subtilis, the Bacillus subtilis DB104 can be used and, as a cloning vector, the plasmid pUB18-P43 can be used.

However, since the host-vector system for Escherichia coli has the advantages that it contains various kind of vectors and transformation can be carried out simply and highly effectively, as many as possible procedures for constructing an expression vector are desirably, if possible, carried out by using Escherichia coli. That is, in Escherichia coli, an optional nucleotide sequence containing a termination codon is inserted between two protease gene fragments derived from the plasmid pTC4 and the plasmid pTCS6 so that the full length protease TCES gene is not reconstituted, thus, making expression of the

gene product impossible and, therefore, the construction of a plasmid can be carried out. Then, this inserted sequence can be removed at the final stage to make the expression plasmid pSTC3 of interest shown in Fig. 10.

The procedures for constructing the plasmid pSTC3 shown in Fig. 9 are explained below.

First, the about 1.8 kb HindIII-SspI fragment inserted into the plasmid pTCS6 is inserted between the HindIII site and the EcoRV site of the plasmid vector pBR322 (manufactured by Takara Shuzo Co., Ltd.) to make the recombinant plasmid pBTC5 and, from this plasmid, the DNA fragment between the HindIII site and the KpnI site derived from a multicloning site of the plasmid vector pUC118 and the BamHI site present on the plasmid vector pBR322 are removed to make the plasmid pBTC5HKB.

Then, based on the nucleotide sequence of the protease TCES gene, the primer TCE12 which can introduce the EcoRI site and the BamHI site in front of an initiation codon of the protease TCES, and the primer TCE20R which can introduce the ClaI site and a termination codon on the 3′ side of only one SacI site present in the nucleotide sequence are synthesized. SEQ ID Nos. 18 and 19 of the Sequence Listing show the nucleotide sequences of the primer TCE12 and the primer TCE20R, respectively.

An about 0.9 kb DNA fragment which has been

amplified by PCR using a chromosomal DNA of Thermococcus celer as a template and using these two primers is digested with EcoRI and ClaI (manufactured by Takara Shuzo Co., Ltd.), and inserted into between the EcoRI site and the ClaI site of the plasmid pBTC5HKB to obtain the plasmid pBTC6, which has a mutant gene where the nucleotide sequence of 69 bp long including a termination codon is inserted into the SacI site of the protease TCES gene.

A ribosome binding site derived from the Bacillus subtilis P43 promoter [J. Biol. Chem., volume 259, page 8619-8625 (1984)] is introduced between the KpnI site and the BamHI site of the plasmid vector pUC18 to make the plasmid pUC-P43. The nucleotide sequences of the synthetic oligonucleotides BS1 and BS2 are shown in SEQ ID Nos. 20 and 21 of the Sequence Listing, respectively. Then, the plasmid pBTC6 is digested with BamHI and SphI (both manufactured by Takara Shuzo Co., Ltd.) to obtain an about 3 kb DNA fragment containing a mutant gene of the protease TCES, which is inserted between the BamHI site and the SphI site of the plasmid pUC-P43 to construct the plasmid pTC12.

All the above procedures for constructing a plasmid can be carried out using Escherichia coli as a host.

The SacI site present in the plasmid vector pUC18-P43 used for cloning into Bacillus subtilis is

previously removed, and an about 3 kb KpnI-SphI DNA fragment obtained from the pTC12 can be inserted into between the KpnI site and the SphI site to make the plasmid pSTC2 using Bacillus subtilis DB104 as a host. The plasmid contains a mutant gene of the protease TCES having the P43 promoter and a ribosome binding site sequence on its 5′ side. After the plasmid pSTC2 is digested with SacI, and intramolecular ligation is carried out to obtain a recombinant plasmid, from which the inserted sequence contained in the SacI site of the above mutant gene has been removed. The recombinant plasmid was designated the plasmid pSTC3, and Bacillus subtilis DB104 transformed with the plasmid was designated Bacillus subtilis DB104/pSTC3 and has been deposited at National Institute of Bioscience and Human-Technology at 1-1-3, Higashi, Tsukuba-shi, Ibaraki-ken, Japan under accession number FERM BP-5635 since December 1, 1995 (original deposit date) according to Budapest Treaty. The transformant is cultured, and a culture supernatant and an extract from the cells were investigated for the protease activity. As a result, the thermostable protease activity is found in both samples.

Fig. 10 shows a restriction map of the plasmid pSTC3. In Fig. 10, a thick solid line designates the DNA fragment inserted into the plasmid vector pUB18-P43.

When the amino acid sequences of the protease

PFUL, the protease TCES and subtilisin are aligned so that the regions having the homology coincide with each other as shown in Figs. 11 and 12, it is seen that the protease PFUL has the regions which is not homologous with the sequence of the protease TCES and that of subtilisin at the C-terminal side thereof as well as between the regions having the homology. From this, it is contemplated that, besides the protease PFUL, a protease having a smaller molecular weight than that of the protease PFUL, such as the protease TCES or subtilisin may be present in Pyrococcus furiosus. In order to search a gene encoding such a protease, southern hybridization can be carried out using a chromosomal DNA prepared from Pyrococcus furiosus as a target, and using a DNA fragment containing the nucleotide sequence within the protease TCES gene, which encoding the amino acid sequence which is well conserved in three proteases, for example, the about 150 bp DNA fragment inserted into the plasmid p1F-2R (2), as a probe.

Although, since the DNA fragment used for a probe has also the homology with the protease PFUL gene, the gene fragment is detected as a signal depending upon the hybridization conditions, the position of the signal derived from the gene can be previously estimated on each restriction enzyme used for cutting a chromosomal DNA, from the informations on the nucleotide sequence of the protease PFUL gene and

the restriction map. When some enzymes are used, in addition to the position predicted on the protease PFUL gene, an another signal is detected as almost the same level, suggesting the possibility that at least one protease is present in _Pyrococcus furiosus_ in addition to the protease PFUL.

For isolating a gene corresponding to the above new signal, a portion of the gene is first cloned so as to prevent the failure of isolation of the gene, as in a case of the protease TCES, resulted from the expression of the gene product which is harmful or lethal to _Escherichia coli_. For example, when a chromosomal DNA of _Pyrococcus furiosus_ is digested with the restriction enzymes SacI and SpeI (both manufactured by Takara Shuzo Co., Ltd.) and the digestion products are used to conduct southern hybridization as described above using a fragment of the protease TCES gene as a probe, it was revealed that a new signal corresponding to about 0.6 kb, derived from the new gene, was observed replacing with a signal corresponding to about 3 kb which was observed in a case of digestion with only SacI. This about 0.6 kb SpeI-SacI fragment encodes the amino acid sequence of at maximum around 200 residues and it can not be contemplated to express a protease having the activity. A _Pyrococcus furiosus_ chromosomal DNA digested with SacI and SpeI is subjected to agarose gel

electrophoresis to recover a DNA fragment corresponding to about 0.6 kb from the gel.

Then, the fragment is inserted between the SpeI site and the SacI site of the plasmid vector pBluescript SK(-) (manufactured by Stratagene) and the resulting recombinant plasmid is used to transform <u>Escherichia coli</u> JM109. From this transformant, a clone with a fragment of interest incorporated can be obtained by colony hybridization using the same probe as that used for the above southern hybridization. Whether a plasmid contained in the resulting clone has the sequence encoding a protease or not can be confirmed by conducting PCR using the primers 1FP1, 1FP2, 2RP1 and 2RP2 (the nucleotide sequences of the primers 1FP1, 1FP2, 2RP1 and 2RP2 are shown in SEQ ID Nos. 22, 23, 24, and 25 of the Sequence Listing) made based on the amino acid sequence common to the above various proteases, or by determining the nucleotide sequence of a DNA fragment inserted into the plasmid prepared from the clone. The plasmid in which the existence of a protease gene is confirmed in this manner was designated the plasmid pSS3. The nucleotide sequence of a DNA fragment inserted in the plasmid, and the amino acid sequence deduced therefrom are shown in SEQ ID No. 26 of the Sequence Listing.

The amino acid sequence deduced from the

nucleotide sequence of the DNA fragment inserted into the plasmid pSS3 is shown to have the homology with the sequences of subtilisin, the protease PFUL, the protease TCES and the like. A product of a protease gene different from the protease PFUL, a portion of which was obtained newly from Pyrococcus furiosus, was designated the protease PFUS. A region encoding a N-terminal side part of the protease, that is, a region 5′ of the SpeI site, and a region encoding a C-terminal side part, that is, a gene fragment 3′ of the above SacI site can be obtained by the inverse PCR method. If the restriction enzyme sites in the protease PFUS gene and the vicinity thereof in a chromosome are revealed in advance, the inverse PCR can be carried out using an appropriate restriction enzyme. The restriction enzyme sites can be revealed by cutting a chromosomal DNA of Pyrococcus furiosus with various restriction enzymes, and conducting southern hybridization using a DNA fragment inserted into the plasmid pSS3 as a probe. Consequently, it is shown that the SacI site is located on about 3 kb distant 5′ side of the SpeI site and the XbaI site is located on about 5 kb distant 3′ side of the SacI site.

A primer used for the inverse PCR can be design to anneal at around an end of the SpeI-SacI fragment contained in the plasmid pSS3. The primers designed to anneal at around the SacI site are designated NPF-1 and NPF-2 and a

primer designed to anneal at around the SpeI site is designated NPR-3. The nucleotide sequences thereof are shown in SEQ ID Nos. 27, 28 and 29 of the Sequence Listing, respectively.

A chromosomal DNA of Pyrococcus furiosus is digested with SacI or XbaI (both manufactured by Takara Shuzo Co., Ltd.), respectively, which is allowed to intramolecullarly ligate, and this reaction mixture can be used as a template for the inverse PCR. When a chromosomal DNA is digested with SacI, an about 3 kb fragment is amplified by the inverse PCR, which is inserted into the plasmid vector pT7BlueT (manufactured by Novagen) to obtain a recombinant plasmid which was designated the plasmid pS322. On the other hand, in a case of a chromosomal DNA digested with XbaI, an about 9 kb fragment is amplified. The amplified fragment is digested with XbaI to obtain an about 5 kb fragment which is recovered and inserted into the plasmid vector pBluescript SK(-) to obtain a recombinant plasmid, which was designated the plasmid pSKX5. By combining the results of southern hybridization performed using the SpeI-SacI fragment contained in the plasmid pSS3 as a probe, and those of analysis on the plasmids pS322 and pSKX5 with the restriction enzymes, a restriction map of the protease PFUS gene and the vicinity thereof in a chromosome can be obtained. The restriction

map is shown in Fig. 13.

In addition, by analyzing the nucleotide sequence on a 5′ fragment inserted into the plasmid pS322 in a 5′ direction starting from the SpeI site, the amino acid sequence of an enzyme protein encoded by the region can be deduced. The resulting nucleotide sequence and the amino acid sequence deduced therefrom are shown in SEQ ID No. 30 of the Sequence Listing. Since the amino acid sequence of this region has the homology with that of a protease such as subtilisin or the like, an initiation codon of the protease PFUS can be presumed based on this homology and, thus, primer NPF-4 which can introduce the BamHI site in front of the initiation codon of the protease PFUS can be designed. On the other hand, the nucleotide sequence determined by analyzing the nucleotide sequence of a 3′ fragment of the protease PFUS gene inserted into the plasmid pSKX5 in a 5′ direction starting from the XbaI site is shown in SEQ ID No. 31 of the Sequence Listing. Based on the nucleotide sequence, the primer NPR-4 which can insert the SphI site into the vicinity of the XbaI site can be designed. The nucleotide sequences of the primers NPF-4 and NPR-4 are shown in SEQ ID Nos. 32 and 33 of the Sequence Listing, respectively. The full length protease PFUS gene can be amplified by using these two primers and using a chromosomal DNA of <u>Pyrococcus</u> <u>furiosus</u> as a

template.

The protease PFUS can be expressed in the Bacillus subtilis system, as in a case of the protease TCES. A plasmid for expressing the protease PFUS can be constructed based on the expression plasmid pSTC3 for the protease TCES. First, a DNA fragment containing the full length protease PFUS gene which can be amplified by the PCR is digested with BamHI and SacI to recover an about 0.8 kb fragment encoding a N-terminal part of the enzyme. And this fragment is replaced with the BamHI-SacI fragment, also encoding a N-terminal part of the protease TCES, of the plasmid pSTC3. The resulting expression plasmid encoding a hybrid protein of the protease TCES and the protease PFUS gene was designated the plasmid pSPT1. Fig. 14 shows a restriction map of the plasmid pSPT1.

Then, the above PCR-amplified DNA fragment is digested with SpeI and SphI to give an about 5.7 kb fragment which is isolated and replaced with the SpeI-SphI fragment encoding a C-terminal part of the protease TCES in the plasmid pSPT1. The expression plasmid thus constructed was designated the plasmid pSNP1, and Bacillus subtilis DB104 transformed with the plasmid was designated Bacillus subtilis DB104/pSNP1 and has been deposited at National Institute of Bioscience and Human-Technology (NIBH) at 1-1-3, Higashi, Tsukuba-shi, Ibaraki-ken, Japan since

December 1, 1995 (original deposit date) as the accession number FERM BP-5634 under Budapest Treaty. Fig. 15 shows a restriction map of the plasmid pSNP1.

The Bacillus subtilis DB104/pSNP1 is cultured and a culture supernatant and an extract from the cells are examined for the protease activity and it is found that the thermostable protease activity is found in both samples.

The nucleotide sequence of a gene encoding the protease PFUS can be determined by digesting a DNA fragment inserted into the plasmid pSNP1 with a restriction enzyme into the appropriate sized fragments, subcloning the fragments into an appropriate cloning vector, and conducting the dideoxy method using the subcloned fragments as a template. SEQ ID No. 34 of the Sequence Listing shows the nucleotide sequence of open reading frame present in the nucleotide sequence thus obtained. In addition, SEQ ID No. 35 of the Sequence Listing shows the amino acid sequence of the protease PFUS deduced from the nucleotide sequence.

Further, also when Bacillus subtilis DB104 transformed with the plasmid pSPT1, Bacillus subtilis DB104/pSPT1, is cultured, the protease activity is found in both a culture supernatant and an extract from the cells. SEQ ID No. 6 of the Sequence Listing shows the nucleotide sequence of open reading frame encoding a hybrid protein of

the protease TCES and the protease PFUS. In addition, SEQ ID No. 5 of the Sequence Listing shows the amino acid sequence of the hybrid protein deduced from the nucleotide sequence.

An amount of an expressed protease of the present invention can be increased by utilizing a gene which is highly expressed in Bacillus subtilis, particularly a secretory protein gene. As such a gene, the genes of α-amylase and the various extracellular proteases can be used. For example, an amount of the expressed protease PFUS can be increased by utilizing the promoter and the signal sequence of subtilisin. That is, by ligating the full length protease PFUS gene to downstream of a region encoding the signal sequence of subtilisin gene so that the translation frames of both genes coincide with each other, the protease PFUS can be expressed as a fusion protein under the control of subtilisin gene promoter.

As the promoter and the signal sequence of subtilisin, those of subtilisin gene, which are inserted into the plasmid pKWZ, described in J. Bacteriol., volume 171, page 2657-2665 (1989) can be used. The nucleotide sequence of the gene is described in the above literature for a 5′ upstream region containing the promoter sequence and in J. Bacteriol., volume 158, page 411-418 (1984) for a region encoding subtilisin, respectively. Based on these

sequences, the primer SUB4 for introducing the EcoRI site upstream of the promoter sequence of the gene, and the primer BmRI for introducing the BamHI site behind a region encoding the signal sequence of subtilisin are synthesized, respectively. SEQ ID Nos. 36 and 37 of the Sequence Listing show the nucleotide sequences of the primers SUB4 and BmR1, respectively. By using the primers SUB4 and BmR1, an about 0.3 kb DNA fragment containing the region encoding from the promoter to the signal sequence of subtilisin gene can be amplified by PCR using the plasmid pKWZ as a template.

The protease PFUS gene ligated downstream of the DNA fragment can be taken from a chromosomal DNA of Pyrococcus furiosus by the PCR method. As a primer which hybridizes with a 5′ part of the gene, the primer NPF-4 can be used. In addition, a primer which hybridizes with a 3′ part can be made after the nucleotide sequence downstream of a termination codon of the gene is determined. That is, a portion of the nucleotide sequence of the plasmid pSNPD obtained by subcloning an about 0.6 kb fragment, produced by digestion of the plasmid pSNP1 with BamHI, into the BamHI site of the plasmid vector pUC119 is determined (the nucleotide sequence is SEQ ID No. 38 of the Sequence Listing). Based on the sequence, the primer NPM-1 which hybridizes with a 3′ part of the protease PFUS gene and

which can introduce the SphI site is synthesized. SEQ ID No. 39 of the Sequence Listing shows the sequence of the primer NPM-1.

On the other hand, when the protease PFUS gene is ligated to the above 0.3 kb DNA fragment by utilizing the BamHI site, only one BamHI site present in the gene becomes a barrier to the procedures. The primers mutRR and mutFR for removing this BamHI site by the PCR-mutagenesis method can be made based on the nucleotide sequence of the protease PFUS gene shown in SEQ ID No. 34 of the Sequence Listing. The nucleotide sequences of the primers mutRR and mutRF are shown in SEQ ID Nos. 40 and 41, respectively. In addition, when the BamHI site is removed by utilizing these primers, glycine present at the position 560 in the amino acid sequence of the protease PFUS shown in SEQ ID No. 35 of the Sequence Listing is substituted with valine due to the nucleotide substitution which is introduced into the site.

By using these primers, the protease PFUS gene to be ligated to the promoter to signal sequence-coding region of subtilisin gene can be obtained. That is, two kinds of PCRs are carried out using a chromosomal DNA of Pyrococcus furiosus as a template and using two kinds of pairs of the primers mutRR and NPF-4, and the primers mutFR and NPM-1. Further, the second PCR is carried out using a hetero

duplex formed by mixing the DNA fragments amplified by both PCRs as a template, and using the primers NPF-4 and NPM-1. Thus, the full length of the about 2.4 kb protease PFUS gene containing no BamHI site can be amplified.

An about 2.4 kb DNA fragment obtained by digesting the above PCR-amplified DNA fragment with BamHI and SphI is isolated, and replaced with the BamHI-SphI fragment containing the protease PFUS gene in the plasmid pSNP1. The expression plasmid thus constructed was designated pPS1 and Bacillus subtilis DB104 transformed with the plasmid was designated Bacillus subtilis DB104/pPS1. When the transformant is cultured, the similar protease activity to that in a case of the use of the plasmid pSNP1 is found in both a culture supernatant and an extract from the cells, and it is confirmed that the substitution of the amino acids dose not affect on the enzyme activity. Fig. 16 shows a restriction map of the plasmid pPS1.

An about 0.3 kb DNA fragment containing from the promoter to the signal sequence of the subtilisin is digested with EcoRI and BamHI, and substituted with the EcoRI-BamHI fragment containing the P43 promoter and the ribosome binding site in the plasmid pPS1. The expression plasmid thus constructed was designated pNAPS1 and Bacillus subtilis transformed with the plasmid was designated Bacillus subtilis DB104/pNAPS1. The transformant is

cultured, a culture supernatant and an extract from the cells are examined for the protease activity to be found that the protease activity is recognized in both samples. An amount of expressed enzyme is increased as compared with Bacillus subtilis DB104/pSN1. Fig. 17 shows a restriction map of the plasmid pNAPS1.

By a similar method to that in a case of the protease TCES gene and the protease PFUS gene, a protease gene having the homology with these genes can be obtained from hyperthermophiles other than Pyrococcus furiosus and Thermococcus celer. However, in PCR using the above oligonucleotides PRO-1F, PRO-2F, PRO-2R and PRO-4R as a primer and using a chromosomal DNA of Staphylothermus marinus DSM3639 and that of Thermobacteroides proteoliticus DSM5265 as a template, the amplification of a DNA fragment as found in Thermococcus celer was not found.

In addition, it is known that the efficiency of gene amplification by PCR is largely influenced by the efficiency of annealing of a 3′ terminal part of a primer and a template DNA. Even when the amplification of a DNA by PCR is not observed, a protease gene can be detected by synthesizing and using the oligonucleotides having the different nucleotide sequence from that used this time but encoding the same amino acid sequence. Alternatively, a protease gene can be also detected by conducting southern

hybridization using a chromosomal DNA and using the above oligonucleotides or a portion of other hyperthermostable protease genes as a probe.

An about 1 kb DNA fragment encoding the sequence of residue 323 to residue 650 of the amino acid sequence of the protease PFUL represented by SEQ ID No. 8 of the Sequence Listing is prepared, and this can be used as a probe to conduct genomic southern hybridization using a chromosomal DNA of Staphylothermus marinus DSM3639 and that of Thermobacteroides proteoliticus DSM5265. As a result, when the Staphylothermus marinus chromosomal DNA digested with PstI (manufactured by Takara Shuzo Co., Ltd.) is used, a signal is observed at the position of about 4.8 kb. On the other hand, when the Thermobacteroides proteoliticus chromosomal DNA digested with XbaI is used, a signal is observed at the position of about 3.5 kb.

From this, it is revealed that a sequence having the homology with the protease PFUL, the protease PFUS and the protease TCES gene is present also in the Staphylothermus marinus and Thermobacteroides proteoliticus DNA chromosomes. From the DNA fragment thus detected, a gene encoding a hyperthermostable protease present in Staphylothermus marinus or Thermobacteroides proteoliticus can be isolated and identified by using the same method as that when the gene encoding the protease TCES or the

protease PFUS is isolated and identified.

The transformant in which the protease TCES gene, a hyperthermostable protease gene of the present invention, is introduced (<u>Bacillus subtilis</u> DB104/pSTC3) expresses a hyperthermostable protease in a culture by culturing at 37 °C in LB medium containing 10 µg/ml kanamycin. After the completion of cultivation, crude enzyme preparation is obtained by subjecting centrifugation of a culture to collect a supernatant, and salting out with ammonium sulfate and dialysis. Thus, the crude enzyme preparation obtained from <u>Bacillus subtilis</u> DB104/pSTC3 was designated TC-3.

According to the similar procedures, a crude enzyme preparation can be obtained from the transformant <u>Bacillus subtilis</u> DB101/pSNP1 in which the protease PFUS gene is introduced, or from the transformant <u>Bacillus subtilis</u> DB104/pSPT1 in which a gene encoding a hybrid protease of the protease TCES and the protease PFUS. Crude enzyme preparations obtained from <u>Bacillus subtilis</u> DB104/pSNP1 and <u>Bacillus subtilis</u> DB104/pSPT1 were designated NP-1 and PT-1, respectively.

Transformant <u>Bacillus subtilis</u> DB104/pNAPS1 in which the protease PFUS gene, a hyperthermostable protease gene of the present invention, is introduced expresses a hyperthermostable protease in the cells or culture under

the conventional conditions, for example, by culturing at 37 °C in LB medium containing 10 µg/ml kanamycin. After the completion of cultivation, the cells and a culture supernatant are separated by centrifugation, from either of which a crude enzyme preparation of the protease PFUS can be obtained by the following procedures.

When an enzyme is purified from the cells, the cells are first lysed by the lysozyme treatment, the lysate is heat-treated and centrifuged to recover a supernatant. This supernatant can be fractionated with ammonium sulfate and subjected to hydrophobic chromatography to obtain a purified enzyme. The purified enzyme preparation thus obtained from Bacillus subtilis DB104/pNAPS1 was designated NAPS-1.

On the other hand, the culture supernatant is dialyzed and subjected to anion-exchange chromatography. The eluted active fractions can be collected, heat-treated, fractionated with ammonium sulfate, and subjected to hydrophobic chromatography to obtain a purified enzyme of the protease PFUS. The purified enzyme preparation was designated NAPS-1S.

When the purified products NAPS-1 and NAPS-1S thus obtained are subjected to SDS-polyacrylamide gel electrophoresis, both enzyme preparation show a single band corresponding to a molecular weight of about 45 kDa. These

two enzyme preparation are substantially the same enzyme preparation which have been converted into a mature (active-type) enzyme by removing a pro sequence by heat-treatment during the purification procedures.

The protease preparation produced by the transformants in which a hyperthermostable protease gene obtained by the present invention is introduced, for example, TC-3, NP-1, PT-1, NAPS-1 and NAPS-1S have the following enzymatic and physicochemical properties.

(1) Activity

The enzymes obtained in the present invention hydrolyze gelatin to produce the short-chain polypeptides. In addition, the enzymes hydrolyze casein to produce short-chain polypeptides.

Sub B3

In addition, the enzymes obtained in the present invention hydrolyze succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine-4-methylcoumarin-7-amide (Suc-Leu-Leu-Val-Tyr-MCA) to produce a fluorescent material (7-amino-4-methylcoumarin).

Further, the enzymes obtained in the present invention hydrolyze succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-p-nitroanilide (Suc-Ala-Ala-Pro-Phe-p-NA) to produce a yellow material (p-nitroaniline).

(2) Method for measuring enzyme activity

The enzyme activity of the enzyme preparations

obtained in the present invention can be measured using a synthetic peptide substrate.

Sub B4

The enzyme activity of the enzyme preparation TC-3 obtained in the present invention can be measured using as a substrate Suc-Leu-Leu-Val-Tyr-MCA (manufactured by Peptide Laboratory). That is, the enzyme preparation to be detected for the enzyme activity is appropriately diluted, to 20 μl of the solution is added 80 μl of a 0.1M sodium phosphate buffer (pH 7.0) containing 62.5 μM Suc-Leu-Leu-Val-Tyr-MCA, followed by incubating at 75 °C for 30 minutes. After the reaction is stopped by the addition of 20 μl of 30% acetic acid, the fluorescent intensity is measured at the excitation wavelength of 355 nm and the fluorescence wavelength of 460 nm to quantitate an amount of the generated 7-amino-4-methylcoumarin, and the resulting value is compared with that obtained when incubating without the addition of the enzyme preparation, to investigate the enzyme activity. The enzyme preparation TC-3 obtained by the present invention had the Suc-Leu-Leu-Val-Tyr-MCA hydrolyzing activity measured at pH 7.0 and 75 °C.

In addition, the enzyme activity of the enzyme preparations NP-1, PT-1, NAPS-1 and NAPS-1S can be photometrically measured using Suc-Ala-Ala-Pro-Phe-p-NA (manufactured by Sigma) as a substrate. That is, an enzyme

preparation to be detected for the enzyme activity was appropriately diluted, to 50 μl of the solution was added 50 μl of a 0.1M potassium phosphate buffer (pH 7.0) containing Suc-Ala-Ala-Pro-Phe-p-NA (Suc-Ala-Ala-Pro-Phe-p-NA solution), followed by incubating at 95 °C for 30 minutes. After the reaction was stopped by ice-cooling, the absorbance at 405 nm was measured to quantitate an amount of the generated p-nitroaniline, and the resulting value was compared with that when incubating without the addition of the enzyme preparation, to investigate the enzyme activity. Upon this, a 0.2 mM solution of Suc-Ala-Ala-Pro-Phe-p-NA was used for the enzyme preparations NP-1 and PT-1 and a 1 mM solution was used for the enzyme preparations NAPS-1 and NAPS-1S. The enzyme preparations NP-1, PT-1, NAPS-1 and NAPS-1S obtained by the present invention have the Suc-Ala-Ala-Pro-Phe-p-NA hydrolyzing activity at measured pH 7.0 and 95 °C.

(3) Detection of activity on various substrates

The activity of the enzyme preparations obtained in the present invention on the synthetic peptide substrates is confirmed by a method for measuring the enzyme activity described in the above (2). That is, the enzyme preparation TC-3 obtained in the present invention has the Suc-Leu-Leu-Val-Tyr-MCA hydrlyzing activity, and the enzyme preparations NP-1, PT-1, NAPS-1 and NAPS-1A have the

Suc-Ala-Ala-Pro-Phe-p-NA hydrlyzing activity, respectively. In addition, the enzyme preparations NP-1, PT-1, NAPS-1 and NAPS-1S were investigated for the Suc-Leu-Leu-Val-Tyr-MCA hydrlyzing activity by the enzyme activity measuring method described in the above (2) used for the enzyme preparation TC-3, and it was shown that these enzyme preparations had the activity to degrade the substrates. Further, the enzyme preparation TC-3 was investigated for the Suc-Ala-Ala-Pro-Phe-p-NA hydrlyzing activity by the enzyme activity measuring method described in the above (2) used for the enzyme preparations NP-1 and PT-1, and the activity to degrade the substrate was recognized. In addition, the activity of the enzyme preparations obtained in the present invention on gelatin can be detected by confirming the degradation of gelatin by an enzyme on the SDS-polyacrylamide gel. That is, the enzyme preparation to be detected for the enzyme activity was appropriately diluted, to 10 µl of the sample solution was added 2.5 µl of a sample buffer (50 mM Tris-HCl, pH 7.5, 5% SDS, 5% 2-mercaptoethanol, 0.005% Bromophenol Blue. 50% glycerol), followed by treatment at 100 °C for 5 minutes and electrophoresis using 0.1% SDS-10% polyacrylamide gel containing 0.05% gelatin. After the completion of run, the gel was soaked in a 50 mM potassium phosphate buffer (pH 7.0), and incubated at 95 °C for 3 hours to carry out the

Sub B3

enzyme reaction. Then, the gel was stained in 2.5% Coomassie Brilliant Blue R-250, 25% ethanol and 10% acetic acid for 30 minutes, and transferred in 7% acetic acid to remove the excess dye over 3 to 15 hours. The presence of the protease activity was detected by the fact that gelatin is hydrolyzed by a protease into peptides which are diffused out of the gel and, consequently, the relevant portion of the gel was not stained with Coomassie Brilliant Blue. The enzyme preparations TC-3, NP-1, PT-1, NAPS-1 and NAPS-1S obtained by the present invention had the gelatin hydrolyzing activity at 95 °C.

In addition, the enzyme preparations NP-1, NAPS-1 and NSPA-1S derived from the protease PFUS gene are recognized to have the gelatin hydrlyzing activity at the almost same positions on the gel in the above activity measuring method. From this, it is shown that, in these enzyme preparations, the processing from a precursor enzyme into a mature type enzyme occurs in the similar manner.

Further, the hydrlyzing activity on casein can be detected according to the same method as that used for detecting the activity on gelatin except that 0.1% SDS-10% polyacrylamide gel containing 0.05% casein is used. The enzyme preparations TC-3, NP-1, PT-1, NAPS-1 and NAPS-1S obtained by the present invention had the casein hydrolyzing activity at 95 °C.

Alternatively, the casein hydrolyzing activity of the enzyme preparations TC-3, NP-1, NAPS-1 and NAPS-1S obtained by the present invention can be measured by the following method. 100 μl of an appropriately diluted enzyme preparation was added to 100 μl of a 0.1M potassium phosphate buffer (pH 7.0) containing 0.2% casein, incubated at 95 °C for 1 hour, and the reaction was stopped by the addition of 100 μl of 15% trichloroacetic acid. An amount of an acid-soluble short-chain polypeptide contained in the supernatant obtained by centrifugation of this reaction mixture was determined from the absorbance at 280 nm and compared with that when incubating without the addition of an enzyme preparation, to investigate the enzyme activity. The enzyme preparations TC-3, NP-1, NAPS-1 and NAPS-1S obtained by the present invention had the casein hydrolyzing activity at 95 °C.

(4) Optimum temperature

The optimum temperature of the enzyme preparation TC-3 obtained by the present invention was investigated using the enzyme activity measuring method shown in the above (2) except for varying a temperature. As shown in Fig. 18, the enzyme preparation TC-3 showed the activity at a temperature of 37 to 95 °C and the optimum temperature thereof was 70 to 80 °C. That is, Fig. 18 is a figure showing the relationship between the activity of the enzyme

preparation TC-3 obtained in the present invention and a temperature, and the ordinate shows the relative activity to the maximum activity (%) and the abscissa shows a temperature.

In addition, the optimum temperature of the enzyme preparation NAPS-1 obtained in the present invention was investigated by using the enzyme activity measuring method shown in the above (2) except for varying a temperature. As shown in Fig. 19, the enzyme preparation NAPS-1 had the activity at a temperature between 40 to 110 °C at the measuring conditions of pH 7.0, and the optimum temperature being 80 to 95 °C. That is, Fig. 19 is a figure showing the relationship between the activity of the enzyme preparation NAPS-1 obtained in the present invention and a temperature, and the ordinate shows the relative activity to the maximum activity (%) and the abscissa shows a temperature.

(5) Optimum pH

Sub B4

~~The optimum pH of the enzyme preparation TC-3 obtained by the present invention was investigated by the enzyme activity measuring method shown in the above (2). That is, the Suc-Leu-Leu-Val-Tyr-MCA solutions were prepared using the buffers having various pHs, and the enzyme activities obtained by using these solutions were compared. As a buffer, a sodium acetate buffer was used at~~

pH 3 to 6, a sodium phosphate buffer at pH 6 to 8, a sodium borate buffer at pH 8 to 9, and a sodium phosphate-sodium hydroxide buffer at pH 10 to 11. As shown in Fig. 20, the enzyme preparation TC-3 shows the activity at pH 5.5 to 9, and the optimum pH was pH 7 to 8. That is, Fig. 20 is a figure showing the relationship between the activity of the enzyme preparation TC-3 obtained in the present invention and pH, and the ordinate shows the relative activity (%) and the abscissa shows pH.

Sub B4

In addition, the optimum pH of the enzyme preparation NP-1 obtained in the present invention was investigated by the enzyme activity measuring method shown in the above (2). That is, the Suc-Ala-Ala-Pro-Phe-pNA solutions were prepared by using the buffers having various pHs, and the enzyme activities obtained by using these solution were compared. As a buffer, a sodium acetate buffer was used at pH 4 to 6, a potassium phosphate at pH 6 to 8, a sodium borate buffer at pH 5 to 10, and a sodium phosphate-sodium hydroxide buffer at pH 10.5. As shown in Fig. 21, the enzyme preparation NP-1 shows the activity at pH 5 to 10, and the optimum pH was pH 5.5 to 8. That is, Fig. 21 is a figure showing the relationship between the activity of the enzyme preparation NP-1 obtained in the present invention and pH, and the ordinate shows the relative activity (%) and the abscissa shows pH.

Further, the optimum pH of the enzyme preparation NAPS-1 obtained in the present invention was investigated by the enzyme activity measuring method shown in the above (2). That is, the Suc-Ala-Ala-Pro-Phe-pNA solutions were prepared by using the buffers having various pHs, and the enzyme activities obtained by using these solution were compared. As a buffer, a sodium acetate buffer was used at pH 4 to 6, a potassium phosphate at pH 6 to 8, a sodium borate buffer at pH 8.5 to 10. As shown in Fig. 22, the enzyme preparation NAPS-1 shows the activity at pH 5 to 10, and the optimum pH was pH 6 to 8. That is, Fig. 22 is a figure showing the relationship between the activity of the enzyme preparation NAPS-1 obtained in the present invention and pH, and the ordinate shows the relative activity (%) and the abscissa shows pH.

(6) Thermostability

The thermostability of the enzyme preparation TC-3 obtained by the present invention was investigated. That is, the enzyme preparation was incubated at 80 °C in 20 mM Tris-HCl, pH 7.5 for various periods of time, an appropriate amount thereof was taken to measure the enzyme activity by the method shown in the above (2), and the activity was compared with that when not heat-treated. As shown in Fig. 23, the enzyme preparation TC-3 obtained by the present invention had not less than 90% of the activity

even after the heat-treatment for 3 hours and, thus, was stable on the above heat-treatment. That is, Fig. 23 is a figure showing the thermostability of the enzyme preparation TC-3 obtained in the present invention, and the ordinate shows the residual activity (%) after the heat-treatment and the abscissa shows time.

In addition, the thermostability of the enzyme preparation NP-1 obtained in the present invention was investigated. That is, the enzyme preparation was incubated at 95 °C in 20 mM Tris-HCl, pH 7.5 for various periods of time, an appropriate aliquot was taken to determine the enzyme activity by the method shown in the above (2), and the enzyme activity was compared with that when not heat-treated. As shown in Fig. 24, the enzyme preparation NP-1 obtained in the present invention is observed to have the remarkably increased enzyme activity when incubated at 95 °C. This is considered to be because a protease produced as a precursor causes the self-catalytic activation during the heat-treatment. In addition, no decrease in the activity was recognized in the heat-treatment for up to 3 hours. That is, Fig. 24 is a figure showing the thermostability of the enzyme preparation NP-1 obtained in the present invention, and the ordinate shows the residual activity (%) after the heat-treatment and the abscissa shows time.

In addition, the above enzyme preparation NP-1 activated by the heat-treatment was investigated for the thermostability. That is, the enzyme preparation NP-1 was activated by the heat-treatment at 95 °C for 30 minutes, incubated at 95 °C for various periods of time, and the activity was determined as described above to compare with that when not heat-treated. At the same time, buffers having the various pHs (sodium acetate buffer at pH 5, potassium phosphate buffer at pH 7, sodium borate buffer at pH 9, sodium phosphate-sodium hydroxide buffer at pH 11, 20 mM in every case) were used. As shown in Fig. 25, when the activated enzyme preparation NP-1 obtained in the present invention was treated in a buffer at pH 9, it had not less than 90% of the activity after the heat-treatment for 8 hours and approximately 50% of the activity even after the heat-treatment for 24 hours and, thus, being very stable to the above heat-treatment. That is, Fig. 25 is a figure showing the thermostability of the enzyme preparation NP-1 obtained in the present invention, and the ordinate shows the residual activity (%) after the heat-treatment and the abscissa shows time.

In addition, the enzyme preparation NAPS-1 obtained by the present invention was investigated for the thermostability. That is, a temperature of the enzyme preparation was maintained at 95 °C in 20 mM Tris-HCl, pH

7.5 for various periods of time, an appropriate aliquot was taken to determine the enzyme activity by the method shown in the above (2) to compare with that when not heat-treated. As shown in Fig. 26, the enzyme preparation NAPS-1 obtained by the present invention had not less than 80% of the activity even after the heat-treatment at 95 °C for 3 hours and, thus, being stable against the above heat-treatment. That is, Fig. 26 is a figure showing the thermostability of the enzyme preparation NAPS obtained in the present invention, and the ordinate shows the residual activity (%) after the heat-treatment and the abscissa shows time.

(7) pH stability

The pH stability of the enzyme preparation NP-1 obtained by the present invention was investigated according to the following procedures. Each 50 μl of 20 mM buffers at various pHs, which contain the enzyme preparation NP-1 activated by the heat-treatment at 95 °C for 30 minutes, was treated at 95 °C for 60 minutes, and an appropriate aliquot was taken to determine the enzyme activity by the method shown in the above (2) to compare with that when not treated. As a buffer, a sodium acetate buffer was used at pH 4 to 6, a potassium phosphate buffer at pH 6 to 8, a sodium borate buffer at pH 9 to 10, a sodium phosphate-sodium hydroxide buffer at pH 11. As

shown in Fig. 27, the enzyme preparation NP-1 obtained by the present invention retained not less than 95% of the activity even after the treatment at 95 °C for 60 minutes at pH between 5 and 11. That is, Fig. 27 is a figure showing the pH stability of the enzyme obtained by the present invention, and the ordinate shows the residual activity (%) and abscissa shows pH.

(8) Stability to detergent

The stability to detergent of the enzyme preparation NP-1 obtained by the present invention was investigated using SDS as detergent. The enzyme preparation NP-1 was activated by the heat-treatment at 95 °C for 30 minutes. Each 50 µl of a solution containing only the enzyme preparation and a solution further containing SDS to the final concentration of 0.1% or 1% was prepared. These solutions were incubated at 95 °C for various periods of time, an appropriate amount thereof was taken to determine the enzyme activity by the method described in the above (2) to compare with that when not treated. As shown in Fig. 28, the activated enzyme preparation NP-1 obtained by the present invention had not less than 80% of the activity after the heat-treatment at 95 °C for 8 hours and approximately 50% of the activity even the after heat-treatment for 24 hours independently of the presence of SDS and, thus, having the high stability

even in the presence of SDS. That is, Fig. 28 is a figure showing the stability to SDS of the enzyme preparation NP-1 obtained by the present invention, and the ordinate shows the residual activity (%) and the abscissa shows time.

In addition, the stability to detergent of the enzyme preparation NAPS-1 obtained by the present invention was investigated using SDS as detergent. Each 50 μl of a solution containing only the enzyme preparation NAPS-1 and a solution further containing SDS to the final concentration of 0.1% or 1% was prepared. These solutions was incubated at 95 °C for various periods of time, an appropriate aliquot was taken to determine the enzyme activity by the method described in the above (2) to compare with that when not treated. As shown in Fig. 29, the enzyme preparation NAPS-1 obtained by the present invention had approximately 80% of the activity after the heat-treatment at 95 °C for 3 hours independently of the presence of SDS. That is, Fig. 29 is a figure showing the stability to SDS of the activated enzyme preparation NAPS-1 obtained by the present invention, and the ordinate shows the residual activity (%) and the abscissa shows time.

When the above results are compared, it is shown that the enzyme preparation NAPS-1 has remarkably decreased residual activity in comparison with the enzyme preparation NP-1. However, this phenomenon is hardly considered to be

even in the presence of SDS. That is, Fig. 28 is a figure showing the stability to SDS of the enzyme preparation NP-1 obtained by the present invention, and the ordinate shows the residual activity (%) and the abscissa shows time.

In addition, the stability to detergent of the enzyme preparation NAPS-1 obtained by the present invention was investigated using SDS as detergent. Each 50 µl of a solution containing only the enzyme preparation NAPS-1 and a solution further containing SDS to the final concentration of 0.1% or 1% was prepared. These solutions was incubated at 95 °C for various periods of time, an appropriate aliquot was taken to determine the enzyme activity by the method described in the above (2) to compare with that when not treated. As shown in Fig. 29, the enzyme preparation NAPS-1 obtained by the present invention had approximately 80% of the activity after the heat-treatment at 95 °C for 3 hours independently of the presence of SDS. That is, Fig. 29 is a figure showing the stability to SDS of the activated enzyme preparation NAPS-1 obtained by the present invention, and the ordinate shows the residual activity (%) and the abscissa shows time.

When the above results are compared, it is shown that the enzyme preparation NAPS-1 has remarkably decreased residual activity in comparison with the enzyme preparation NP-1. However, this phenomenon is hardly considered to be

based on the difference in the stability to SDS of the enzyme proteins themselves contained in both preparations. It is thought to be the cause for the above phenomenon that NAPS-1 which is the purified enzyme preparation has less contaminant proteins as compared with NP-1 and, thereby, the inactivation easily occurs due to self-digestion.

(9) Stability to organic solvent

The stability to an organic solvent of the enzyme preparation NAPS-1 obtained by the present invention was investigated using acetonitrile. Each 50 μl of enzyme preparation NAPS-1 solutions containing acetonitrile to the final concentration of 25% or 50% was incubated at 95 °C for various periods of time, and an appropriate aliquot was taken to determine the activity by the method described in the above (2) to compare with that when not treated. As shown in Fig. 30, the enzyme preparation NAPS-1 obtained by the present invention had the activity of not less than 80% of that before the treatment, even after the treatment at 95 °C for 1 hour in the presence of 50% acetonitrile. That is, Fig. 30 is a figure showing the stability to acetonitrile of the enzyme preparation NAPS-1 obtained by the present invention.

(10) Stability to denaturing agent

The stability to various denaturing agents of the enzyme preparation NAPS-1 obtained by the present invention

was investigated using urea and guanidine hydrochloride. Each 50 μl of the enzyme preparation NAPS-1 solution containing urea to the final concentration of 3.2 M or 6.4 M or guanidine hydrochloride to the final concentration of 1 M, 3.2 M or 6.4 M was prepared. These solutions were incubated at 95 °C for various periods of time, an appropriate aliquot was taken to determine the activity by the method described in the above (2) to compare with that when not treated. As shown Fig. 31, the enzyme preparation NAPS-1 obtained by the present invention shows the resistance to urea and had the activity of not less than 70% of that before the treatment, even after the treatment at 95 °C for 1 hour in the presence of 6.4 M urea. That is, Fig. 31 is a figure showing the stability to urea and Fig. 32 is a figure showing the stability to guanidine hydrochloride, and the ordinate indicates the residual activity and the abscissa indicates time.

(11) Effects of various reagents

The effects of various reagents on the enzyme preparations TCES and NAPS-1 obtained by the present invention were investigated. That is, the above enzyme preparations were treated at 37 °C for 30 minutes in the presence of the various reagents at the final concentration of 1 mM, and an aliquot thereof was taken to determine the enzyme activity by the method described in the above (2) to

compare with that (control) when no reagent was added. The results are shown in Table 1.

Table 1

Reagent	TCES	NAPS-1
Control	100%	100%
EDTA	103.5%	36.1%
PMSF	8.1%	0.1%
Antipain	19.0%	81.9%
Chymostatin	0%	6.6%
Leupeptin	104.5%	89.3%
Pepstatin	105.2%	100.7%
N-ethylmaleimide	82.6%	102.6%

As shown in Table 1, when treated with PMSF (phenylmethanesulfonyl fluoride) and chymostatin, both enzyme preparations had the remarkably decreased activity. In addition, when treated with antipain, the decrease in the activity was observed in TCES, and when treated with EDTA, in NAPS-1, respectively. In a case of other reagents, the large decrease was not observed in the activity.

(12) Molecular weight

A molecular weight of the enzyme preparation NAPS-1 obtained by the present invention was determined by SDS-PAGE using 0.1% SDS-10% polyacrylamide gel. The enzyme preparation NAPS-1 showed a molecular weight of about 45 kDa on SDS-PAGE. On the other hand, the enzyme preparation NAPS-1S showed the same molecular weight as that of the

enzyme preparation NAPS-1.

(13) N-terminal amino acid sequence

The N-terminal amino acid sequence of a mature enzyme, the protease PFUS, was determined using the enzyme preparation NAPS-1 obtained by the present invention. The enzyme preparation NAPS-1 electrophoresed on 0.1% SDS-10% polyacrylamide gel was transferred onto the PVDF membrane, and the N-terminal amino acid sequence of the enzyme on the membrane was determined by the automated Edman degradation using a protein sequencer. The N-terminal amino acid sequence of the mature type protease PFUS thus determined is shown in SEQ ID No. 42 of the Sequence Listing. The sequence coincided with the sequence of amino acids 133 to 144 in the amino acid sequence of the protease PFUS represented by SEQ ID No. 35 of the Sequence Listing, and it was shown that the mature protease PFUS is an enzyme consisting of the polypeptides including behind this part. The amino acid sequence of the mature protease PFUS thus revealed is represented by SEQ ID No. 3 of the Sequence Listing. In addition, as described above, there is no influence on the enzyme activity of the protease PFUS independently of whether 428th amino acid (corresponding to 560th amino acid in the amino acid sequence represented by SEQ ID No. 35 of the Sequence Listing) is glycine or valine. Further, within the nucleotide sequence of the

protease PUFS gene represented by SEQ ID No. 34 of the Sequence Listing, that of a region encoding the mature type enzyme is shown in SEQ ID No. 4. 1283rd base in the sequence may be guanine or thimine.

In a case of in vitro gene amplification by PCR, the misincorporation of a nucleotide may occur during the elongation reaction, leading to the nucleotide substitution in the sequence of the resulting DNA. This frequency largely depends upon the kind of the enzyme used for PCR, the composition of the reaction mixture, the reaction conditions, the nucleotide sequence of a DNA to be amplified and the like. However, when a certain region in a gene is simply amplified as performed usually, the frequency is at best around one nucleotide per 400 nucleotides. In the present invention, PCR was used for isolation of a gene of the protease TCES or the protease PFUS or construction of the expression plasmid therefor. The number of nucleotide substitutions in the nucleotide sequence of the resulting gene is, if any, a few nucleotides. Taking into consideration the fact that the nucleotide substitution on a gene dose not necessarily lead to the amino acid substitution in the expressed protein due to degeneracy of translation codons, the number of the possible amino acid substitutions can be evaluated to be at best 2 to 3 in the whole residues. It cannot be denied

that the nucleotide sequence of a gene of the protease TCES and the protease PFUS and the amino acid sequence of the proteases disclosed herein are different from natural ones. However, the object of the present invention is to disclose a hyperthermostable protease having the high activity at high temperature and a gene encoding the same and, therefore, the protease and the gene are not limited to the same enzyme and the same gene encoding the same as the natural ones. And it is clear to those skilled in the art that even a gene having the possible nucleotide substitution can hybridize to a natural gene under the stringent conditions.

Further, in the specification, a method for obtaining a gene of interest is clearly disclosed such that (1) the library for expression cloning is made from a chromosomal DNA of the hyperthermophiles and the expression of the protease activity is screened, (2) a gene possibly expressing the hyperthermostable protease is isolated by hybridization or PCR based on the homology of amino acid sequences, and the enzyme action of expression products of these genes, that is, the hyperthermostable protease activity is confirmed using an appropriate microorganism. Therefore, it can be easily determined by using the above method whether the gene sequence with the mutation introduced encodes a hyperthermostable protease, after a

variety of mutations are introduced into the hyperthermostable protease gene of the present invention using the known mutation introducing method. The kind of the mutation to be introduced is not limited to specified ones as long as the gene sequence obtained as a result of the mutation introduction expresses substantially the same protease activity as that of the hyperthermostable protease of the present invention. However, in order that the expressed protein retains the protease activity, the mutation is desirably introduced into a region other than four regions which are conserved in common in the serine proteases.

A mutation can be randomly introduced into any region of a gene encoding the hyperthermostable protease (random mutagenesis), or alternatively, a desired mutation can be introduced into a specified pre-determined position (site-directed mutagenesis). As a method for randomly introducing a mutation, for example, there is a method for chemically treating a DNA. In this case, a plasmid is prepared such that a region into which a mutation is sought to be introduced is partially single-stranded, and sodium bisulfite is acted on this partially single-stranded region to convert a base cytosine into uracil and, thus, introducing a transition mutation from C:G to T:A. In addition, a method for producing a base substitution during

a process where a single-stranded part is repaired to double-strand in the presence of [α-S] dNTP is also known. The details of these methods are described in Proc. Natl. Acad. Sci. USA, volume 79, page 1408-1412 (1982), and Gene, volume 64, page 313-319 (1988).

Random mutation can also be introduced by conducting PCR under the conditions where fidelity of the nucleotide incorporation becomes lower. In particular, the addition of manganese to the reaction system is effective and the details of this method are described in Anal. Biochem., volume 224, page 347-355 (1995). As a method for introducing a site-directed mutation, for example, there is a method using a system where a gene of interest is made single-stranded, a primer designed depending upon a mutation sought to be introduced in this single-stranded part is synthesized, and the primer is annealed to the part, which is introduced into in vivo system where only the strand with a mutation introduced is selectively replicated. The details of this method are described in Methods in Enzymology, volume 154, page 367 (1987). For example, a mutation introducing kit, Mutant K manufactured by Takara Shuzo Co., Ltd. can be used. Site-directed mutagenesis can be conducted also by PCR and the details are described of the method in PCR Technology, page 61-70 (1989), edited by Ehlich and published by Takara Shuzo Co.,

Ltd. Alternatively, for example, LA-PCR in vitro mutagenesis kit manufactured by Takara Shuzo Co., Ltd. can be used. By using the above method, a mutation of substitution, deletion and insertion can be introduced.

Thus, an enzyme having the similar thermostability and optimum temperature to those of the hyperthermostable protease of the present invention but having a little different, for example, optimum pH can be produced in a host by introducing a mutation using as a base the hyperthermostable protease gene of the present invention. In this case, the base nucleotide sequence of the hyperthermostable protease gene is not necessarily limited to the sequence derived from one hyperthermostable protease.

A hybrid gene can be made by recombinating two or more hyperthermostable protease genes having a sequence homologous to each other, such as those disclosed by the present invention, by exchanging the homologous sequence, and the hybrid enzyme encoded by the gene can be produced in a host. Also in a case of a hybrid gene, whether it is a hyperthermostable protease gene can be determined by testing for the enzyme action of the gene product, that is, the protease activity. For example, by using the above plasmid pSPT1, a hybrid protease of which N-terminal part is derived from the protease PFUS and of which C-terminal

part is derived from the protease TCES can be produced in Bacillus subtilis, and this hybrid protease has the protease activity at 95 °C.

The hybrid enzyme is expected to have the properties of two or more base enzymes at the same time. For example, when the protease TCES and the protease PFUS disclosed herein are compared, the protease TCES is superior in respect of the extracellular secretion efficiency and the protease PFUS is superior in respect of the thermostability. Since a signal sequence located at a N-terminal of the proteins has the great influence on extracellular secretion efficiency, if an expression plasmid is constructed so that a protein having, in contrast with pSPT1, a N-terminal part derived from the protease TCES and a C-terminal part derived from the protease PFUS is produced, a hyperthermostable protease having the equal thermostability to that of the protease PFUS can be secreted at the equal secretional efficiency to that of the protease TCES. In addition, since a signal sequence is cut from an enzyme when the enzyme is extracellularly secreted, it has little influence on the nature of the enzyme itself. Therefore, when a hyperthermostable protease is produced using a mesophile, its signal sequence dose not necessarily need to be derived from hyperthermophiles and a signal sequence derived from

a mesophile has no problem as long as a protein of interest is extracellularly secreted at a higher efficiency.

In particular, when a signal sequence of a secretory protein which is highly expressed in a host to be used is employed, a higher secretion is expected.

Upon construction of the above hybrid gene, a recombination dose not necessarily need to be conducted site-directedly. Alternatively, a hybrid gene can be made, for example, by mixing two or more DNAs of a hyperthermostable protease gene, which are raw materials for construction of the hybrid gene, fragmenting these with a DNA degrading enzyme and reconstituting these fragments using a DNA polymerase. The details of this method are described in Proc. Natl. Acad. Sci. USA, volume 91, page 10747-10751 (1994). Also in this case, a sequence of a gene encoding a hyperthermostable gene can be isolated and identified from the resulting hybrid genes by examining the hyperthermostable protease activity of expressed proteins as described above. In addition, it is expected that sequences encoding four regions common to the serine proteases are conserved in the sequences of the genes thus obtained.

Therefore, it is clear to those skilled in the art that the resulting hybrid gene can hybridize to a DNA selected from the oligonucleotides PRO-1F, PRO-2F, PRO-2R

and PRO-4R having the nucleotide sequences represented by SEQ ID Nos. 9, 10, 11 and 12 of the Sequence Listing by the appropriate hybridization conditions. In addition, it is also clear that a novel hyperthermostable protease gene obtained by the above mutation introduction can hybridize to a gene having a DNA sequence selected from nucleotide sequences represented by SEQ ID Nos. 9, 10, 11 and 12 of the Sequence Listing, for example, the protease PFUL gene by the appropriate hybridization conditions.

In the specification, we described by focusing on obtaining of a hyperthermostable gene. However, a gene encoding a novel protease having both high thermostability and other properties can be made by constructing a hybrid gene of the hyperthermostable protease gene of the present invention and a protease gene having a sequence homology with the hyperthermostable protease gene of the present invention but having no thermostability, for example, by constructing a hybrid gene with a gene of subtilisin to improve the thermostability of subtilisin, to obtain a gene encoding a protease having the properties originally retained by subtilisin and the higher thermostability.

In the present invention, we used Escherichia coli and Bacillus subtilis as a host into which a gene is introduced in order to detect the protease activity retained by a protein encoded by a gene and produce an

enzyme preparation. However, hosts into which a gene is introduced are not limited to specified ones. Any hosts can be used as long as a transforming method is established for the hosts, such as _Bacillus_ _brevis_, _Lactobacillus_, yeast, mold fungi, animal cells, plant cells, insect cells and the like. Upon this, it is important that a polypeptide is folded such that an expressed protein becomes an active form and this does not result in the harmful or lethal effect. Among hosts listed above, _Bacillus_ _brevis_, _Lactobacillus_ and mold fungi which are known to secret their products in a medium can be used as a host for mass production of a protease of interest on an industrial scale, in addition to _Bacillus_ _subtilis_.

Examples

The following Examples further describe the present invention in detail but are not limit the scope thereof.

Example 1

(1) Preparation of oligonucleotide for detection of hyperthermostable protease gene

By comparing the amino sequence of the protease PFUL represented by SEQ ID No. 8 of the Sequence Listing with those of alkaline serine proteases derived from the known bacterium, the homologous amino acid sequences common

to them proved to exist. Among them, three regions were selected and the oligonucleotides were designed, which were used as primers for PCR to detect hyperthermostable protease genes.

Figs. 2, 3 and 4 show the relationship among the amino acid sequences corresponding to the above three regions of the protease PFUL, the nucleotide sequences of the protease PFUL gene encoding the regions, and the nucleotide sequences of the oligonucleotides PRO-1F, PRO-2F, PRO-2R and PRO-4R synthesized based thereon. SEQ ID Nos. 9, 10, 11 and 12 show the nucleotide sequences of the oligonucleotides PRO-1F, PRO-2F, PRO-2R and PRO-4R, respectively.

(2) Preparation of chromosomal DNA of *Thermococcus celer*

10 ml of a culture of *Thermococcus celer* DSM2476 obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH was centrifuged to collect the cells which were suspended in 100 µl of 50 mM Tris-HCl, pH 8.0 containing 25% sucrose. To this suspension was added 20 µl of 0.5 M EDTA and 10 µl of 10 mg/ml lysozyme, and was incubated at 20 °C for 1 hour, 800 µl of a SET solution (150 mM NaCl, 1mM EDTA, 20mM Tris-HCl, pH 8.0), 50 µl of 10% SDS and 10 µl of 20 mg/ml proteinase K were added thereto, and was incubated at 37 °C for 1 hour. The

reaction was stopped by extraction with phenol-chloroform and precipitated with ethanol to recover a DNA which was dissolved in 50 μl of a TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) to give a chromosomal DNA solution.

(3) Detection of hyperthermostable protease gene by PCR

A PCR reaction mixture was prepared from the above chromosomal DNA of Thermococcus celer and the oligonucleotides PRO-1F and PRO-2R, or PRO-2F and PRO-4R, and a 35 cycles reaction was carried out, each cycle consisting of 94 °C for 1 minute - 55 °C for 1 minute - 72 °C for 1 minute. When an aliquot of these reaction mixture were subjected to agarose gel electrophoresis, amplification of three DNA fragments in case of the using the oligonucleotides PRO-1F and PRO-2R, and one DNA fragments in case of the using the oligonucleotides PRO-2F and PRO-4R were observed. These amplified fragments were recovered from the agarose gel, and the DNA ends thereof were made blunt using a DNA blunting kit (manufactured by Takara Shuzo Co., Ltd.) and phosphorylated using the T4 polynucleotide kinase (manufactured by Takara Shuzo Co., Ltd.). Then, the plasmid vector pUC19 (manufactured by Takara Shuzo Co., Ltd.) was digested with HincII (manufactured by Takara Shuzo Co., Ltd.), the resulting fragments were dephosphorylated at ends thereof by alkaline

phosphatase (manufactured by Takara Shuzo Co. Ltd.), mixed with the above PCR-amplified DNA fragments to allow to ligate, followed by introduction into <u>Escherichia</u> <u>coli</u> JM109. Plasmids were prepared from the resulting transformant, and the plasmids with an appropriate size DNA fragment inserted were selected, followed by sequencing of the inserted fragment by the dideoxy method.

Of these plasmids, the amino acid sequence deduced from the nucleotide sequence of the plasmid p1F-2R(2) containing an about 150 bp DNA fragment amplified using the oligonucleotides PRO-1F and PRO-2R, and that deduced from the nucleotide sequence of the plasmid p2F-4R containing an about 550 bp DNA fragment amplified using oligonucleotides PRO-2F and PRO-4R contained sequences having the homology with the amino acid sequences of the protease PFUL, subtilisin and the like. SEQ ID No. 13 of the Sequence Listing shows the nucleotide sequence of the inserted DNA fragment in the plasmid p1F-2R(2) and the amino acid sequence deduced therefrom and SEQ ID NO. 14 of the Sequence Listing shows the nucleotide sequence of the inserted DNA fragment in the plasmid p2F-4R and the amino acid sequence deduced therefrom. In the nucleotide sequence represented by SEQ ID No. 13 of the Sequence Listing, the sequence of 1st to 21st nucleotides and that of 113rd to 145th nucleotides and, in the nucleotide

sequence represented by SEQ ID No. 14 of the Sequence Listing, the sequence of 1st to 32nd nucleotides and that of 532nd to 564th nucleotides are the sequences of the oligonucleotides (corresponding to oligonucleotides PRO-1F, PRO-2R, PRO-2F and PRO-4R, respectively) used as primers for PCR.

Fig. 5 shows a figure of a restriction map of the plasmid p2F-4R.

(4) Screening of protease gene derived from Thermococcus celer

The chromosomal DNA of Thermococcus celer was partially digested with the restriction enzyme Sau3AI (manufactured by Takara Shuzo Co., Ltd.), followed by partial repair of the DNA ends using Klenow Fragment (manufactured by Takara Shuzo Co., Ltd.) in the presence of dATP and dGTP. The DNA fragments were mixed with the lambda GEM-11 XhoI Half-Site Arms Vector (manufactured by Promega) to allow to ligate, which was subjected in vitro packaging using Gigapack Gold (manufactured by Stratagene) to prepare a lambda phage library containing the chromosomal DNA fragments of Thermococcus celer. A part of the library was transformed into Escherichia coli LE392 (manufactured by Promega) to form the plaques on a plate, and the plaques were transferred to Hybond-N+ membrane (manufactured by Amersham). After transference, the

membrane was treated with 0.5N NaOH containing 1.5M NaCl, then with 0.5M Tris-HCl, pH 7.5 containing 3M NaCl, washed with 6 × SSC, air dried, and irradiated with ultraviolet rays on the UV transilluminator to fix the phage DNA to the membrane.

On the other hand, the plasmid p2F-4R was digested with PmaCI and StuI (both manufactured by Takara Shuzo Co., Ltd.), which was subjected to 1% agarose gel electrophoresis to recover the separated about 0.5 kb DNA fragment. By using this fragment as a template and using Random Primer DNA Labeling Kit Ver.2 (manufactured by Takara Shuzo Co., Ltd.) and [α-^{32}P]dCTP (manufactured by Amersham), a ^{32}P-labeled DNA probe was prepared.

The membrane with the DNA fixed thereto was treated with a hybridization buffer (6 × SSC containing 0.5% SDS, 0.1% SBA, 0.1% polyvinylpyrrolidone, 0.1% Ficoll 400, 0.01% denatured salmon sperm DNA) at 50 °C for 2 hours, and transferred to the same buffer containing the ^{32}P-labeled DNA probe, followed by hybridization at 50 °C for 15 hours. After the completion of hybridization, the membrane was washed with 2 × SSC containing 0.5% SDS at room temperature, then with 1 × SSC containing 0.5% SDS at 50 °C. The membrane was further rinsed with 1 × SSC, air dried and a X-ray film was exposed thereto at -80 °C for 6 hours to obtain an autoradiogram. About 3,000 phage clones

were screened and, as a result, one clone containing a protease gene was obtained. Based on the signal on the autoradiogram, the position of this phage clone was found and the plaque corresponding on the plate used for transfer to the membrane was isolated into 1 ml of a SM buffer (50 mM Tris-HCl, pH 7.5, 1M NaCl, 8 mM MgSO4, 0.01% gelatin) containing 1% chloroform.

(5) Detection of phage DNA fragment containing protease gene derived from <u>Thermococcus</u> <u>celer</u>

Transduced <u>Escherichia</u> <u>coli</u> LE392 using the above phage clone was cultured in the NZCMY medium (manufactured by Bio101) at 37 °C for 15 hours to obtain a culture, from which a supernatant was collected to prepare a phage DNA using QIAGEN-lambda kit (manufactured by QIAGEN). The resulting phage DNAs were digested with BamHI, EcoRI, EcoRV, HincII, KpnI, NcoI, PstI, SacI, SalI, SmaI and SphI (all manufactured by Takara Shuzo Co., Ltd.), respectively, followed by agarose gel electrophoresis. Then, DNAs were transferred from the gel to Hybond-N+ membrane according to the southern transfer method described in Molecular Cloning; A Laboratory Manual, 2nd edition (1986), edited by T. Maniatis, et al., published by Cold Spring Harbor Laboratory.

The resulting membrane was treated in a hybridization buffer at 50 °C for 4 hours, and transferred

to the same buffer containing the ^{32}P-labeled DNA probe used in Example 1-(4), followed by hybridization at 50 °C for 18 hours. After the completion of hybridization, the membrane was washed in 1 × SSC containing 0.5% SDS at 50 °C, then rinsed with 1 × SSC and air dried. The membrane was exposed to a X-ray film at -80 °C for 6 hours to obtain an autoradiogram. This autoradiogram indicated that an about 9 kb DNA fragment contained a protease gene in case of the phage DNA digested with KpnI.

Then, the phage DNA containing the above protease gene was digested with KpnI, and further digested successively with BamHI, PstI and SphI, followed by 1% agarose gel electrophoresis. According to the similar procedures to those described above, southern hybridization was conducted and it was indicated that an about 5 kb KpnI-BamHI fragment contained a protease gene.

(6) Cloning of DNA fragment containing protease gene derived from Thermococcus celer

The phage DNA containing the above protease gene was digested with KpnI and BamHI, which was subjected to 1% agarose gel electrophoresis to separate and isolate an about 5 kb DNA fragment from the gel. Then, the plasmid vector pUC119 (manufactured by Takara Shuzo Co., Ltd.) was digested with KpnI and BamHI, which was mixed with the above about 5 kb DNA fragment to allow to ligate, followed

by introduction into Escherichia coli JM109. Plasmids were prepared form the resulting transformant, the plasmid containing the about 5 kb DNA fragment was selected and designated the plasmid pTC3.

Fig. 6 shows a restriction map of the plasmid pTC3.

(7) Preparation of plasmid pTCS6 containing protease gene derived from Thermococcus celer

The above plasmid pTC3 was digested with SacI, which was electrophoresed using 1% agarose gel, and southern hybridization was carried out according to the same manner as that described in Example 1-(5) for detecting the phage DNA fragment containing a protease gene. A signal on the resulting autoradiogram indicated that an about 1.9 kb DNA fragment obtained by digesting the plasmid pTC3 with SacI contained a hyperthermostable protease gene.

Then, the plasmid pTC3 was digested with SacI, which was subjected to 1% agarose gel electrophoresis to isolate an about 1.9 kb DNA fragment. Then, the plasmid vector pUC118 (manufactured by Takara Shuzo Co., Ltd.) was digested with SacI, which was dephosphorylated using alkaline phosphatase and mixed with the about 1.9 kb fragment to allow to ligate, followed by introduction into Escherichia coli JM109. Plasmids were prepared from the

resulting transformant, and the plasmid containing only one molecule of the about 1.9 kb fragment was selected and designated the plasmid pTCS6.

Fig. 7 shows a restriction map of the plasmid pTCS6.

(8) Determination of nucleotide sequence of DNA fragment derived from Thermococcus celer contained in plasmid pTCS6

In order to determine the nucleotide sequence of the protease gene derived from Thermococcus celer inserted into the plasmid pTCS6, the deletion mutants wherein the DNA fragment portion inserted into the plasmid had been deleted in various length were prepared using Kilo Sequence Deletion Kit (manufactured by Takara Shuzo Co., Ltd.). Among them, several mutants having suitable length of deletion were selected and the nucleotide sequence of each of the inserted DNA fragment parts was determined by the dideoxy method, and these results were combined to determine the nucleotide sequence of the inserted DNA fragment contained in the plasmid pTCS6. SEQ ID No. 15 of the Sequence Listing shows the resulting nucleotide sequence.

(9) Cloning of 5' upstream region of a protease gene derived from Thermococcus celer by PCR using cassette and cassette primer

A 5′ upstream region of the protease gene derived from <u>Thermococcus</u> <u>celer</u> was obtained by using LA PCR in vitro cloning kit (manufactured by Takara Shuzo Co., Ltd.).

Based on the nucleotide sequence of the inserted DNA fragment contained in the plasmid pTCS6 represented by SEQ ID No. 15 of the Sequence Listing, the primer TCE6R for use in cassette PCR was synthesized. SEQ ID No. 16 of the Sequence Listing shows the nucleotide sequence of the primer TCE6R.

Then, a chromosomal DNA of <u>Thermococcus</u> <u>celer</u> was completely digested with HindIII (manufactured by Takara Shuzo Co., Ltd.), and the fragments were ligated to the HindIII cassette (manufactured by Takara Shuzo Co., Ltd.) by the ligation reaction. By using this as a template, a PCR reaction mixture containing the primer TCE6R and the cassette primer C1 (manufactured by Takara Shuzo Co., Ltd.) was prepared, a series of reactions, one cycle of 94 °C for one minute, 30 cycles of 94 °C for 30 seconds - 55 °C for 1 minute - 72 °C for 3 minutes, and one cycle of 72 °C for 10 minutes were carried out. An aliquot of this reaction mixture was subjected to agarose gel electrophoresis and an amplified about 1.8 kb fragment was observed. This amplified fragment was digested with HindIII and SacI, and the about 1.5 kb DNA fragment produced was recovered from the gel after agarose gel electrophoresis. The Hin-

dIII-SacI digested plasmid vector pUC119 was mixed with the above about 1.5 kb DNA fragment to allow to ligate, followed by introduction into Escherichia coli JM109. The plasmid harboured by the resulting transformant was examined, the plasmid with only one molecule of the 1.5 kb fragment inserted was selected and designated the plasmid pTC4.

Fig. 8 shows a restriction map of the plasmid pTC4.

(10) Determination of nucleotide sequence of DNA fragment derived from Thermococcus celer contained in plasmid pTC4 and protease TCES gene

In order to determine the nucleotide sequence of a protease gene derived from Thermococcus celer inserted into the plasmid pTC4, the deletion mutants wherein the DNA fragment portion inserted into the plasmid had been deleted in various length were prepared using Kilo Sequence Deletion Kit. Among them, several mutants having suitable length of deletion were selected and the nucleotide sequence of each of the inserted DNA fragment parts was determined by the dideoxy method, and these results were combined to determine the nucleotide sequence of the inserted DNA fragment contained in the plasmid pTCS4. SEQ ID No. 15 of the Sequence Listing shows the resulting nucleotide sequence.

By combining the sequence with the nucleotide sequence of the inserted DNA fragment contained in the plasmid pTCS6 obtained in Example 1-(8), the whole nucleotide sequence of the protease gene derived from <u>Thermococcus</u> <u>celer</u> was determined. SEQ ID No. 1 and 2 of the Sequence Listing show the nucleotide sequence of open reading frame present in the nucleotide sequence and the amino acid sequence deduced therefrom of the protease derived from <u>Thermococcus</u> <u>celer</u>, respectively. The protease derived from <u>Thermococcus</u> <u>celer</u> encoded by the gene was designated the protease TCES.

(11) Preparation of plasmid pBTC6 containing protease TCES gene

The plasmid pTCS6 was digested with HindIII and SspI (manufactured by Takara Shuzo Co., Ltd.), which was subjected to 1% agarose gel electrophoresis to recover the separated about 1.8 kb DNA fragment. Then, the plasmid vector pBT322 (manufactured by Takara Shuzo Co., Ltd.) was digested with HindIII and EcoRV, which was mixed with the about 1.8 kb DNA fragment to allow to ligate, followed by introduction into <u>Escherichia</u> <u>coli</u> JM109. Plasmids were prepared from the resulting transformant, the plasmid containing only one molecule of the 1.8 kb fragment was selected and designated the plasmid pBTC5.

Then, the plasmid pBTC5 was completely digested

with HindIII and KpnI, which was blunt-ended and was subjected to intramolecular ligation, followed by introduction into <u>Escherichia</u> <u>coli</u> JM109. Plasmids were prepared from the resulting transformant, and the plasmid from which the above two restriction enzyme sites had been removed was selected and designated the plasmid pBTC5HK.

Further, the plasmid pBTC5HK was digested with BamHI, which was blunt-ended, and was subjected to intramolecular ligation, followed by introduction into <u>Escherichia</u> <u>coli</u> JM109. Plasmids were prepared from the resulting transformant, the plasmid from which the BamHI site had been removed was selected and designated the plasmid pBTC5HKB.

The primer TCE12 which can introduce the EcoRI site and the BamHI site in front of an initiation codon on the protease TCES gene, and the primer TCE20R which has 16 bp-long nucleotide sequence complementary to a 3′ part of the SacI site of the plasmid pTCS6 and can introduce the ClaI site and a termination codon were synthesized. SEQ ID Nos. 18 and 19 of the Sequence Listing show the nucleotide sequences of the primer TCE12 and the primer TCE20R, respectively. A PCR reaction mixture was prepared using these two primers and using a chromosomal DNA of <u>Thermococcus</u> <u>celer</u> as a template. A reaction of 25 cycles, each cycle consisting of 94 °C for 30 seconds - 55 °C for

1 minute - 72 °C for 1 minute, was carried out to amplify an about 0.9 kb DNA fragment having these two oligonucleotides on both ends and containing a part of the protease TCES gene.

The above about 0.9 kb DNA fragment was digested with EcoRI and ClaI (manufactured by Takara Shuzo Co., Ltd.), which was mixed with the EcoRI-ClaI digested plasmid pBTC5HKB to allow to ligate, followed by introduction into Escherichia coli JM109. Plasmids were prepared from the resulting transformant, and the plasmid containing only one molecule of the about 0.9 kb fragment was selected and designated the plasmid pBTC6.

(12) Preparation of plasmid pTC12 containing protease TCES gene

The plasmid pBTC6 was digested with BamHI and SphI, which was subjected to 1% agarose gel electrophoresis to recover the separated about 3 kb DNA fragment. Then, the plasmid pUC-P43SD where the ribosome binding site sequence derived from Bacillus subtilis P43 promoter was introduced between the KpnI site and the BamHI site of the plasmid vector pUC18 (manufactured by Takara Shuzo Co., Ltd.) (the nucleotide sequence of the synthetic oligonucleotides BS1 and BS2 used for introduction of the sequence are shown in SEQ ID Nos. 20 and 21 of the Sequence Listing) was digested with BamHI and SphI, which

was mixed with the previously recovered about 3 kb DNA fragment to allow to ligate, followed by introduction into *Escherichia coli* JM109. Plasmids were prepared from the resulting transformant, the plasmid containing only one molecule of the above about 3 kb DNA fragment was selected and designated the plasmid pTC12.

(13) Preparation of plasmid pSTC3 containing protease TCES gene for transforming *Bacillus subtilis*

The above plasmid pTC12 was digested with KpnI and SphI, which was subjected to 1% agarose electrophoresis to recover the separated about 3 kb DNA fragment. Then, the plasmid vector pUB18-P43 was digested with SacI, which was bunt-ended and allowed to self-ligate to give the plasmid vector pUB18-P43S from which the SacI site had been removed. This was digested with KpnI and SphI, which was mixed with the previously recovered about 3kb DNA fragment and allowed to ligate, followed by introduction into *Bacillus subtilis* DB104. Plasmids were prepared from the resulting kanamycin-resistant transformant, and the plasmid containing only one molecule of the above about 3 kb DNA fragment was selected and designated the plasmid pSTC2.

Then, the plasmid pSTC2 was digested with SacI and was subjected to intramolecular ligation, followed by introduction into *Bacillus subtilis* DB104. Plasmids were prepared from the resulting kanamycin-resistant

transformant, the plasmid containing only one SacI site and designated the plasmid pSTC3.

Then, _Bacillus subtilis_ DB104 harbouring the plasmid pSTC3 was designated _Bacillus subtilis_ DB104/pSTC3.

Fig. 10 shows a restriction map of the plasmid pSTC3.

Example 2

(1) Preparation of chromosomal DNA of _Pyrococcus furiosus_

Pyrococcus furiosus DMS3638 was cultured as follows. A medium having the composition of 1% trypton, 0.5% yeast extract, 1% soluble starch, 3.5% Jamarin SÅSolid (manufactured by Jamarin Laboratory), 0.5% Jamarin SÅLiquid (manufactured by Jamarin Laboratory), 0.003% $MgSO_4$, 0.001% NaCl, 0.0001% $FeSO_4Å7H_2O$, 0.0001% $CoSO_4$, 0.0001% $CaCl_2Å7H_2O$, 0.0001% $ZnSO_4$, 0.1 ppm $CuSO_4Å5H_2O$, 0.1 ppm H_3BO_3, 0.1 ppm $KAl(SO_4)_2$, 0.1 ppm $Na_2MoO_4Å2H_2O$, 0.25 ppm $NiCl_2ÅH_2O$ was placed in a 2 liter medium bottle, and was sterilized at 120 °C for 20 minutes, nitrogen gas was blown into the medium to purge out the dissolved oxygen, and the above bacterial strain was inoculated into the medium, followed by

subjecting to stationarily culture at 95 °C for 16 hours. After the completion of cultivation, the cells were collected by centrifugation.

Then, the resulting cells were suspended in 4 ml of 50 mM Tris-HCl (pH 8.0) containing 25% sucrose, to this suspension was added 2 ml of 0.2 M EDTA and 0.8 ml of lysozyme (5 mg/ml) and incubated at 20 °C for 1 hour, 24 ml of a SET solution (150 mM NaCl, 1 mM EDTA, 20mM Tris-HCl, pH 8.0), 4 ml of 5% SDS and 400 µl of proteinase K (10 mg/ml) were added thereto and incubated at 37 °C for another 1 hour. The reaction was stopped by extraction with phenol-chloroform, followed by ethanol precipitation to obtain about 3.2 mg of the chromosomal DNA.

(2) Genomic southern hybridization of Pyrococcus furiosus chromosomal DNA

A chromosomal DNA of Pyrococcus furiosus was digested with SacI, NotI, XbaI, EcoRI and XhoI (all manufactured by Takara Shuzo Co., Ltd.), respectively. An aliquot of the reaction mixture was further digested with SacI and EcoRI, which was subjected to 1% agarose gel electrophoresis, followed by southern hybridization according to the procedures described in Example 1-(5). A ^{32}P-labeled DNA, which was prepared using an about 0.3 kb DNA fragment obtained by digesting the above plasmid p1F-2R(2) with EcoRI and PstI as a template and using BcaBEST DNA Labeling kit (manufacture by Takara Shuzo Co., Ltd.) and [α-^{32}P]dCTP, was used as a probe. A membrane was washed in 2 × SSC containing SDS to the final concentration

of 0.5% at room temperature, rinsed with 2 × SSC and the autoradiogram was obtained. As a result, a signal was observed in two DNA fragments of about 5.4 kb and about 3.0 kb produced by digesting a Pyrococcus furiosus chromosomal DNA with SacI and it was indicated that a protease gene was present on respective fragments. When the SacI-digested fragment was further digested with SpeI (manufactured by Takara Shuzo Co., Ltd.), the signal of the above about 5.4 kb fragment did not show the change but the signal which had been seen in the about 3.0 kb fragment was lost, and a signal was newly observed in the about 0.6 kb fragment. Since the SpeI site is not present in the protease PFUL gene represented by SEQ ID No. 7 of the Sequence Listing, it was suggested that a signal on the about 0.6 kb fragment obtained by the digestion with SacI and SpeI was derived from a novel hyperthermostable protease (hereinafter referred to as "protease PFUS"). In addition, regarding the products from digestion of Pyrococcus furiosus chromosomal DNA with XbaI, a signal was observed on two DNA fragments of about 3.3 kb and about 9.0 kb. From a restriction map of protease PFUL gene shown in Fig. 1, it was presumed that the about 3.3 kb fragment contained the protease PFUL gene and the about 9.0 kb fragment contained the protease PFUS gene. When the above chromosomal DNA was digested with XbaI and SacI, a signal was observed on the

about 2.0 kb fragment and the about 3.0 kb fragment. From the positions of the SacI and XbaI cleavage sites present on the protease PFUL gene shown in SEQ ID No. 7 of the Sequence Listing, it was presumed that the protease PFUL gene is present on the about 2.0 kb SacI-XbaI fragment. On the other hand, it was presumed that the protease PFUS gene was present on the about 3.0 kb fragment. Combining with the results on the digestion with SacI, it was shown that no XbaI site is present on the about 3.0 kb DNA fragment obtained by the digestion with SacI alone.

(3) Cloning of 0.6 kb SpeI-SacI fragment containing protease PFUS gene

A chromosomal DNA of Pyrococcus furiosus was digested with SacI and SpeI, which was subjected to 1% agarose gel electrophoresis to recover the DNA fragment corresponding to about 0.6 kb from the gel. Then, the plasmid pBluescript SK(-) (manufactured by Stratagene) was digested with SacI and SpeI, which was mixed with the about 0.6 kb DNA fragment to allow to ligate, followed by introduction into Escherichia coli JM109 to obtain the plasmid library containing the chromosomal DNA fragments. Transformed Escherichia coli JM109 was seeded on a plate to form the colonies, and the produced colonies were transferred to a Hybond-N+ membrane, which was incubated at 37 °C for about 2 hours on a new LB plate. This membrane

was treated with 0.5N NaOH containing 1.5M NaCl, then with 0.5M Tris-HCl (pH 7.5) containing 1.5 M NaCl, washed with 2 × SSC, air dried and the plasmid DNA was fixed to the membrane by irradiating with ultraviolet rays on a UV transilluminator. This membrane was treated at 50 °C for 2 hours in a hybridization buffer, and transferred to the same buffer containing a ^{32}P-labeled DNA probe used for southern hybridization described in Example 2-(2), to hybridize at 50 °C for 18 hours. After the completion of hybridization, the membrane was washed in 2 × SSC containing 0.5% SDS at room temperature, and washed at 37 °C. Further, the membrane was rinsed with 2 × SSC, air dried, exposed to a X-ray film at -80 °C for 12 hours to obtain an autoradiogram. About 500 clones were screened and, as a result, 3 clones containing a protease gene were obtained. From a signal on the autoradiogram, the positions of these clones were examined and the corresponding colonies on the plate used for transfer to the membrane were isolated in LB medium.

(4) Detection of protease PFUS gene by PCR

Oligonucleotides which used for detection of a hyperthermostable protease gene by PCR as a probe were designed based on the nucleotide sequences encoding two regions having the high homology with the amino acid sequences of alkaline serine proteases derived from the

known microorganisms in the protease PFUL gene. Based on the amino acid sequence of the protease PFUL represented by Figs. 2 and 3, the primers 1FP1, 1FP2, 2RP1 and 2RP2 were synthesized. SEQ ID Nos. 22, 23, 24 and 25 of the Sequence Listing show the nucleotide sequences of the oligonucleotides 1FP1, 1FP2, 2RP1 and 2RP2.

PCR reaction mixtures containing the plasmids prepared from the above three clones as well as the oligonucleotides 1FP1 and 2RP1, or 1FP1 and 2RP2, or 1FP2 and 2RP1, or 1FP2 and 2RP2 were prepared, and a 30 cycle reaction was carried out, each cycle consisting of 94 °C for 30 seconds - 37 °C for 2 minutes -72 °C for 1 minute. It was shown that, when aliquots of these reaction mixtures were subjected to agarose gel electrophoresis, respectively, the amplification of an about 150 bp DNA fragment was observed in all the three above plasmids when used the primers 1FP2 and 2RP2, indicating that a protease gene was present on these plasmids.

One of the above three clones was selected and designated the plasmid pSS3.

(5) Determination of nucleotide sequence of protease PFUS gene contained in plasmid pSS3

The nucleotide sequence of the inserted DNA fragment in the plasmid was determined by the dideoxy method using the plasmid pSS3 as a template and using the

primer M4 and the primer RV (both manufactured by Takara Shuzo Co., Ltd.). SEQ ID No. 26 of the Sequence Listing shows the resultant nucleotide sequence and the amino acid sequence which was deduced to be encoded by the nucleotide sequence. By comparing the amino acid sequence with that of the protease PFUL, the protease TCES and subtilisin, it was presumed that the DNA fragment inserted in the plasmid pSS3 encoded the amino acid sequence having the homology with these proteases.

(6) Cloning of N-terminal coding region and C-terminal coding region of protease PFUS by inverse PCR method

In order to obtain genes encoding N-terminal amino acid sequence and C-terminal one of the protease PFUS, the inverse PCR was carried out. A primer used for the inverse PCR was synthesized based on the nucleotide sequence of the inserted DNA fragment in the plasmid pSS3. SEQ ID Nos. 27, 28 and 29 of the Sequence Listing show the nucleotide sequences of the primers NPF-1, NPF-2 and NPR-3.

A chromosomal DNA of <u>Pyrococcus</u> <u>furiosus</u> was digested with SacI and XbaI and was subjected to intramolecular ligation. PCR mixtures containing an aliquot of the ligation reaction mixture and the primers NPF-1 and NPR-3, or NPF-2 and NPR-3 were prepared and a 30 cycle reaction was carried out, each cycle consisting of 94

°C for 30 seconds - 67 °C for 10 minutes. When an aliquot of this reaction mixture was subjected to agarose gel electrophoresis, an about 3 kb amplified fragment was observed in a case of the use of the primers NPF-2 and NPR-3. This amplified fragment was recovered from the agarose gel, and mixed with the plasmid vector pT7BlueT (manufactured by Novagen) to allow to ligate, followed by introduction into Escherichia coli JM109. Plasmids were prepared from the resultant transformant, the plasmid containing an about 3 kb fragment was selected and designated the plasmid pS322.

On the other hand, an about 9 kb amplified fragment was observed in a case of the use of the primers NPF-1 and NPR-3. This amplified fragment was recovered from the agarose gel, the DNA ends were made blunt using a DNA blunting kit, followed by further digestion with XbaI. This was mixed with the plasmid vector pBluescript SK(-) digested with XbaI and HincII to allow to ligate, followed by introduction into Escherichia coli JM109. Plasmids were prepared from the resulting transformant, the plasmid containing an about 5 kb DNA fragment was selected and designated the plasmid pSKX5.

(7) Sequencing of nucleotide sequence of protease PFUS gene contained in plasmid pS322 and pSKX5

The nucleotide sequence of a gene encoding a

N-terminal region of the protease PFUS was determined by the dideoxy method using the plasmid pS322 as a template and using the primer NPR-3. SEQ ID No. 30 of the Sequence Listing shows a part of the resulting nucleotide sequence and the amino acid sequence deduced to be encoded by the nucleotide sequence.

Further, the nucleotide sequence of a region corresponding to a 3′ part of the protease PFUS gene was determined by the dideoxy method using the plasmid pSKX5 as a template and using the primer RV. SEQ ID No. 31 of the Sequence Listing shows a part of the resulting nucleotide sequence.

(8) Synthesis of primer used for amplification of full length protease PFUS gene

Based on the nucleotide sequence obtained in Example 2-(7), a primer used for amplification of the full length of the protease PFUS gene was designed. Based on the nucleotide sequence encoding a N-terminal part of the protease PFUS shown in SEQ ID No. 30 of the Sequence Listing, the primer NPF-4 which can introduce BamHI site in front of an initiation codon of the protease PFUS gene. SEQ ID No. 32 of the Sequence Listing shows the nucleotide sequence of the primer NPF-4. In addition, based on the nucleotide sequence in the vicinity of a 3′ region of the protease PFUS shown in SEQ ID No. 31 of the Sequence

Listing, the primer NPR-4 having a sequence complementary to the nucleotide sequence and a SphI site was synthesized. SEQ ID No. 33 of the Sequence Listing shows the nucleotide sequence of the primer NPR-4.

(9) Preparation of plasmid pSPT1 containing hybrid gene of protease derived from <u>Pyrococcus</u> <u>furiosus</u> and protease TCES, for transformation of <u>Bacillus</u> <u>subtilis</u>

By using a LA PCR kit (manufactured by Takara Shuzo Co., Ltd.), a PCR reaction mixture (hereinafter a PCR reaction mixture prepared by using a LA PCR kit is referred to as "LA-PCR reaction mixture") containing the primers NPF-4 and NPR-4 and a chromosomal DNA of <u>Pyrococcus</u> <u>furiosus</u>, and a reaction of 30 cycles, each cycle consisting of 94 °C for 20 seconds - 55 °C for 1 minute - 68 °C for 7 minutes, was carried out to amplify an about 6 kb DNA fragment having these two primers on both ends and containing the coding region of the protease PFUS gene.

The about 6 kb DNA fragment was digested with BamHI and SacI, which was subjected to 1% agarose gel electrophoresis to recover the separated about 0.8 kb DNA fragment. This fragment was mixed with the plasmid pSTC3 digested with BamHI and SacI to allow to ligate, followed by introduction into <u>Bacillus</u> <u>subtilis</u> DB104. Plasmids were prepared from the resultant kanamycin-resistant transformant, and the plasmid containing only one molecule

of the above 0.8 kb fragment was selected and designated the plasmid pSPT1.

Bacillus subtilis DB104 harboring the plasmid pSPT1 was designated Bacillus subtilis DB104/pSTP1.

Fig. 14 shows a restriction map of the plasmid pSPT1.

(10) Preparation of plasmid pSNP1 containing protease PFUS gene for transformation of Bacillus subtilis

The about 6 kb DNA fragment amplified in Example 2-(9) was digested with SpeI and SphI, which was subjected to 1% agarose gel electrophoresis to recover the separated about 5.7 kb DNA fragment. This was mixed with the plasmid digested with SpeI and SphI to allow to ligate, followed by introduction into Bacillus subtilis DB104. Plasmids were prepared from the resulting kanamycin-resistant transformant, and the plasmid containing only one molecule of the 5.7 kb fragment was selected and designated the plasmid pSNP1. Bacillus subtilis transformed with the plasmid pSNP1 was designated as Bacillus subtilis DB104/pSNP1.

Fig. 15 shows a restriction map of the plasmid pSNP1.

(11) Determination of nucleotide sequence of protease PFUS gene contained in plasmid pSNP1

An about 6 kb DNA fragment containing a protease

gene inserted into the plasmid pSNP1 was fragmented into appropriate size with a variety of restriction enzymes, and the fragments were subcloned into the plasmid vector pUC119 or pBluescript SK(-). The nucleotide sequence was determined by the dideoxy method using the resulting recombinant plasmid as a template and using a commercially available universal primer. Regarding a part from which the fragments having appropriate size could not be obtained, the primer walking method was used utilizing the synthetic primers. The nucleotide sequence of an open reading frame present in the nucleotide sequence of the DNA fragment inserted into the plasmid pSNP1 thus determined, and the amino acid sequence of a protease derived from Pyrococcus furiosus deduced from the nucleotide sequence are shown in SEQ ID Nos. 34 and 35, respectively.

(12) Synthesis of primer for amplification of protease PFUS gene

In order to design a primer, which is used for amplification of the full length protease PFUS gene and hybridizes to a 3′ part of the gene, the nucleotide sequence of the 3′ part of the gene was determined. First, an about 0.6 kb DNA fragment containing the 3′ region of the protease PFUS gene, obtained by digestion of the plasmid pSNP1 with BamHI, was ligated with the plasmid vector pUC119 which had been digested with BamHI and

dephosphorylated with alkaline phosphatase. The resulting recombinant plasmid was designated the plasmid pSNPD and the nucleotide sequence of a region corresponding to the 3′ part of the protease PFUS gene was determined by the dideoxy method using this as a template. SEQ ID No. 38 of the Sequence Listing shows the nucleotide sequence, from the BamHI site to 80 bp upstream nucleotide, present in the region (the sequence of the complementary chain). Then, based on the sequence, the primer NPM-1 which hybridizes to a 3′ part of the protease PFUS gene and contains a SphI site was synthesized. SEQ ID No. 39 of the Sequence Listing shows the nucleotide sequence of the primer NPM-1.

In addition, the primers mutRR and mutFR for elimination the BamHI sites which are present about 1.7 kb downstream from an initiation codon within the protease PFUS gene were synthesized. SEQ ID Nos. 40 and 41 of the Sequence Listing show the nucleotide sequences of the primers mutRR and mutFR, respectively.

(13) Preparation of plasmid pPS1 containing full length protease PUFS gene

Two sets of LA-PCR reaction mixtures containing <u>Pyrococcus</u> <u>furiosus</u> chromosomal DNA as a template and a combination of the primers NPF-4 and mutRR or a combination of the primers mutFR and NPM-1 were prepared, and a reaction of 30 cycles, each cycle consisting of 94 °C for

30 seconds - 55 °C for 1 minute - 68 °C for 3 minutes, was carried out. When agarose gel electrophoresis was carried out using an aliquot of this reaction mixture, an about 1.8 kb DNA fragment was amplified in a case of the use of the primer NPF-4 and mutRR, and an about 0.6 kb DNA fragment in a case of the use of the primers mutFR and NMP-1.

Each amplified DNA fragment from which the primers had been removed by using SUPREC-02 (manufactured by Takara Shuzo Co., Ltd.) was prepared from the two set of the PCR mixture. A LA-PCR reaction mixture containing both of these amplified DNA fragments and not containing the primers and LA Taq was prepared, which was used to carry out heat denaturation at 94 °C for 10 minutes, followed by cooling to 30 °C over 30 minutes and maintaining at 30 °C for 15 minutes to form a hetero duplex. Then, to this reaction mixture, LA Taq was added and was incubated at 72 °C for 3 minutes, the primers NPF-4 and NPM-1 were added thereto and a reaction of 25 cycles, each cycle consisting of 94 °C for 30 seconds - 55 °C for 1 minute - 68 °C for 3 minutes, was carried out. Amplification of an about 2.4 kb DNA fragment was observed in this reaction mixture.

The about 2.4 kb DNA fragment was digested with BamHI and SphI, the fragments were mixed with the plasmid pSNP1, described in Example 2 -(11), from which the full length protease PFUS gene had been removed previously by

digestion with BamHI and SphI, to allow to ligate, followed by introduction into <u>Bacillus</u> <u>subtilis</u> DB104. Plasmids were prepared from the resulting kanamycin-resistant transformant, and the plasmid with only one molecule of the about 2.4 kb fragment inserted was selected and designated the plasmid pPS1. <u>Bacillus</u> <u>subtilis</u> DB104 transformed with the plasmid DB104 was designated <u>Bacillus</u> <u>subtilis</u> DB104/pPS1.

Fig. 16 shows a restriction map of the plasmid pPS1.

(14) Amplification of DNA fragment of a region from the promoter to the signal sequence of subtilisin gene

A primer for obtaining a region from promoter to signal sequence of subtilisin gene was synthesized. First, with reference to the nucleotide sequence of a promoter region of subtilisin gene described in J. Bacteriol., volume 171, page 2657-2665 (1989), the primer SUB4 which hybridizes to a part upstream of the region and contains the EcoRI site was synthesized (SEQ ID No. 36 of the Sequence Listing shows the nucleotide sequence of the primer SUB4). Then, with reference to the nucleotide sequence of a region encoding subtilisin described in J. Bacteriol., volume 158, page 411-418 (1984), the primer BmR1 which can be introduce the BamHI site just behind the signal sequence was synthesized (SEQ ID No. 37 of the

Sequence Listing shows the nucleotide sequence of the primer BmR1).

The plasmid pKWZ containing subtilisin gene described in J. Bacteriol., volume 17, page 2657-2665 (1989) was used as a template to prepare a PCR reaction mixture containing the primers SUB4 and BmR1, and a reaction of 30 cycles, each cycle consisting of 94 °C for 30 seconds - 55 °C for 1 minute - 68 °C for 2 minutes, was carried out. Agarose gel electrophoresis of an aliquot of this reaction mixture confirmed amplification of an about 0.3 kb DNA fragment.

(15) Preparation of plasmid pNAPS1 containing protease PFUS gene for transformation of <u>Bacillus subtilis</u>

The about 0.3 kb DNA fragment was digested with EcoRI and BamHI, which was mixed with the plasmid pPS1, described in Example 2-(13), which previously had been digested with EcoRI and BamHI to allow to ligate, followed by introduction into <u>Bacillus subtilis</u> DB104. Plasmids were prepared from the resulting kanamycin-resistant transformant and the plasmid containing only one molecule of the about 0.3 kb fragment was selected and designated the plasmid pNAPS1. In addition, <u>Bacillus subtilis</u> DB104 transformed with the plasmid pNAPS1 was designated <u>Bacillus subtilis</u> DB104/pNAPS1.

Fig. 17 shows a restriction map of the plasmid

pNAPS1.

Example 3

(1) Preparation of probe for detecting hyperthermostable protease gene

The plasmid pTPR12 containing the protease PFUL gene was digested with BalI and HincII (both manufactured by Takara Shuzo Co., Ltd.), which was subjected to 1% agarose gel electrophoresis to recover the separated about 1 kb DNA fragment. A ^{32}P-labeled DNA probe was prepared using the DNA fragment as a template and using BcaBEST DNA labeling kit and [α-^{32}P] dCTP.

(2) Detection of hyperthermostable protease gene present in hyperthermophile Staphylothermus marinus and Thermobacteroides proteoliticus

Chromosomal DNAs were prepared from each 10 ml of cultures of Staphylothermus marinus DSM3639 and Thermobacteroides proteoliticus DSM5265 obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH according to the procedures described in Example 1-(3). Both chromosomal DNAs were digested with EcoRI, PstI, HindIII, XbaI and SacI, respectively, which were subjected to 1% agarose gel electrophoresis, followed by southern hybridization according to the procedures described in Example 1-(5). As a probe, ^{32}P-labeled DNA probe prepared in Example 3-(1) was used. A membrane was washed at 37 °C

in 2 × SSC finally containing 0.5% SDS, rinsed with 2 × SSC, and the autoradiogram was obtained. From this autoradiogram, a signal was recognized in an about 4.8 kb DNA fragment in a case of Staphylothermus marinus chromosomal DNA digested with PstI, and in an about 3.5 kb DNA fragment in a case of Thermobacteroides proteoliticus chromosomal DNA digested with XbaI, thus, indicating that a hyperthermostable protease gene which hybridizes with the protease PFUL gene was present in the Staphylothermus marinus and Thermobacteroides proteoliticus chromosomal DNA.

Example 4

(1) Preparation of crude enzyme preparation of protease PFUS and TCES

Bacillus subtilis DB104 in which the plasmid pSTC3 containing the hyperthermostable protease gene of the present invention had been introduced (Bacillus subtilis DB104/pSTC3) was cultured in 5 ml of LB medium (trypton 10 g/liter, yeast extract 5 g/liter, NaCl 5 g/liter, pH 7.2) containing 10 μg/ml kanamycin at 37 °C for 8 hours. 250 ml of the similar medium was prepared in 1 liter Erlenmeyer flask, which was inoculated with 5 ml of the above culture to culture at 37 °C for 16 hours. Ammonium sulfate was added to a supernatant obtained by centrifugation of the culture to 75% saturation, and the resulted precipitates

were recovered by centrifugation. The recovered precipitates were suspended in 4 ml of 20 mM Tris-HCl, pH 7.5, which was dialyzed against the same buffer, and the resulting dialysate was used as crude enzyme preparation (enzyme preparation TC-3).

Crude enzyme preparations were prepared from Bacillus subtilis DB104 in which the plasmid pSNP1 containing the hyperthermostable protease gene of the present invention was introduced (Bacillus subtilis DB104/pSNP1) or Bacillus subtilis DB104 in which the plasmid pSPT1 containing the hyperthermostable protease of the present invention, according to the procedures described above, and the preparations were designated NP-1 and PT-1, respectively.

These enzyme preparations were used to examine the protease activity by the enzyme activity detecting method using the SDS-polyacrylamide gel containing gelatin or by the other activity detecting methods.

(2) Preparation of purified enzyme preparation of protease PFUS

Two tubes containing 5 ml of LB medium containing 10 µl/ml kanamycin were inoculated with Bacillus subtilis DB104 in which the plasmid pNAPS1 containing the hyperthermostable protease gene of the present invention obtained in Example 2-(18) was introduced (Bacillus

subtilis DB104/pNAPS1), followed by cultivation at 37 °C for 7 hours with shaking. Six Erlenmeyer flasks of 500 ml volume, each containing 120 ml of the similar medium, were prepared, and each flask was inoculated with 1 ml of the above culture, followed by cultivation at 37 °C for 17 hours with shaking. The culture was centrifuged to obtain the cells and a culture supernatant.

The cells were suspended in 15 ml of 50 mM Tris-HCl, pH 7.5, and 30 mg of lysozyme (manufactured by Sigma) was added thereto, followed by digestion at 37 °C for 1.5 hours. The digestion solution was heat-treated at 95 °C for 15 minutes, followed by centrifugation to collect a supernatant. To 12 ml of the resulting supernatant was added 4 ml of an saturated ammonium sulfate solution, which was filtrated using 0.45 µm filter unit (Sterivex HV, manufactured by Millipore), and the filtrate was loaded onto the POROS PH column (4.6 mm × 150 mm: manufactured by PerSeptive Biosystems) equilibrated with 25 mM Tris-HCl, pH 7.5 containing ammonium sulfate at 25% saturation. The column was washed with the buffer used for equilibration, the gradient elution was performed by lowering the concentration of ammonium sulfate from 25% saturation to 0% saturation and at the same time increasing the concentration of acetonitrile from 0% to 20% to elute the PFUS protease, to obtain the purified enzyme preparation

NAPS-1.

750 ml of the culture supernatant was dialyzed against 25 mM Tris-HCl, pH 8.0 and adsorbed onto Econo-Pack Q cartridge (manufactured by BioRad) equilibrated with the same buffer. Then, the adsorbed enzyme was eluted with a linear gradient of 0 to 1.5 M NaCl. The resulting active fraction was heat-treated at 95 °C for 1 hour, and an 1/3 volume of a saturated ammonium sulfate solution was added thereto. After the filtration was carried out using a 0.45 μm filter unit (Sterivex HV), the filtrate was loaded onto the POROS PH column (4.6 mm × 150 mm) equilibrated with 25 mM Tris-HCl, pH 7.5 containing ammonium sulfate at 25% saturation. The PFUS protease absorbed onto the column was eluted according to the procedures as in the enzyme preparation NAPS-1 to obtain the purified enzyme preparation NAPS-1.

To an appropriate amount of the purified enzyme preparation NAPS-1 or NAPS-1S was added trichloroacetic acid to the final concentration of 8.3% to precipitate the proteins in the enzyme preparation, which were recovered by centrifugation. The recovered precipitated protein were dissolved in a distilled water, an 1/4 amount of a sample buffer (50 mM Tris-HCl, pH 7.5, 5% SDS, 5% 2-merc-aptoethanol, 0.005% Bromophenol Blue, 50% glycerol) was added thereto, which was treated at 100 °C for 5 minutes

and subjected to electrophoresis using 0.1% SDS-10% polyacrylamide gel. After run, the gel was stained in 2.5% Coomassie Brilliant Blue R-250, 25% ethanol, and 10% acetic acid for 30 minutes, transferred in 25% methanol, and 7% acetic acid and the excess dye was removed over 3 to 15 hours. Both enzyme preparations NAPS-1 and NAPS-1S showed a single band, and a molecular weight deduced from migrated distance was about 4.5 kDa.

(3) Sequencing of N-terminal of mature protease PUFS

The purified enzyme preparation NAPS-1 prepared in Example 4-(2) was subjected to electrophoresis using 0.1% SDS-10% polyacrylamide gel, and the proteins on the gel was blotted onto a PVDF membrane (manufactured by Millipore) using Semidry Blotter (manufactured by Nihon Eido). Blotting was carried out according to a method described in Electrophoresis, volume 11, page 573-580 (1990). After blotting, the membrane was stained with a solution of 1% Coomassie Brilliant Blue R-250, in 50% methanol, and destained with a 60% methanol solution. A part of the membrane which had been stained was cut off, followed by sequencing of the N-terminal amino acid sequence by the automated Edman degradation using G1000A protein sequencer (manufactured by Hewlette Packard). SEQ ID No. 42 shows the resultant N-terminal amino acid sequence.